Molecular Mechanisms Controlling Pluripotency in Embryonic Stem Cells

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CONTENTS

ABBREVIATIONS

4-OHT	4-hydroxy-tamoxifen
ALK	Activin receptor-like kinase
BMP	Bone morphogenetic protein
ChIP	Chromatin immunoprecipitation
ECC	Embryonal carcinoma cell
EGFP	Enhanced green fluorescent protein
ENU	N-ethyl-N-nitrosourea
ERK	Extracellular signal-related kinase
ESC	Embryonic stem cell
FGF	Fibroblast growth factor
gp130	Glycoprotein 130
GSK3	Glycogen synthase kinase 3
hESC	Human embryonic stem cell
ICM	Inner cell mass
Id	Inhibitor of differentiation
iPSC	Induced pluripotent stem cell
JAK	Janus kinase
LIF	Leukemia inhibitory factor
LIFRβ	LIF receptor
MEK or MAPK	Mitogen-activated protein kinase
PI3K	Phosphatidylinositol-3 phosphate kinase
Socs	Suppressor of cytokine signaling
SSEA-1	Stage-specific embryonic antigen-1
STAT3	Signal transducer and activator of transcription
STAT3-ER	STAT3-estrogen receptor
TGF-β	Transforming Growth Factor β

SUMMARY

The transition between morula and blastocyst stage during preimplantation development represents the first differentiation event of embryogenesis. Morula cells undergo the first cellular specialization and produce two well-defined populations of cells, the trophoblast and the inner cell mass (ICM). Embryonic stem cells (ESCs) with unlimited self-renewal capacity are believed to represent the *in vitro* counterpart of the ICM cells.
A unique and complex signaling network allows embryonic stem cells (ESCs) to undergo extended proliferation *in vitro*, while maintaining their capacity for multilineage differentiation. Genuine ESC identity can only be maintained when both self-renewal and suppression of differentiation are active and balanced. Despite many efforts have been made in the past years, aimed at understanding the molecular factors driving and controlling pluripotency *in vivo* and *in vitro*, the precise mechanisms of how these processes are regulated remain largely unknown.
Therefore, the overall goal of this thesis was to gain knowledge on the molecular mechanisms that control stemness in pluripotent stem cells. The work was divided into two different studies: the first was focused on the function of pluripotency related genes in ESCs and the second consisted in a genome wide expression analysis of mouse and rat preimplantation stage embryos.

The activation of the LIF/STAT3 signaling was shown to be sufficient to maintain ESCs in an undifferentiated and pluripotent state. Thus, in a first study the importance of STAT3 in the establishment of ESCs was investigated. In order to identify new STAT3 target genes, ESCs overexpressing STAT3 were derived from a non-permissive mouse strain and their expression profiles were compared with wild type ESCs. Several genes were detected as potential key-players involved in maintenance of pluripotency in ESCs. The function of two of these genes, *Pramel6* and *Pramel7*, was extensively characterized. We identify Pramel7 (preferentially expressed antigen in melanoma-like 7) as a novel factor crucial for maintenance of pluripotency and LIF-mediated self-renewal in ESCs. *In vivo*, *Pramel7* expression was exclusively found in the pluripotent pools of cells, namely, the central part of the morula and the inner cell mass of the blastocyst. Ablation of Pramel7 induced ESC differentiation, whereas its overexpression was sufficient to support long-term self-renewal in the absence of exogenous LIF. Furthermore, Pramel7 overexpression suppressed differentiation in ESCs *in vitro* and *in vivo*. This process was reversible, as on transgene excision cells reverted to a LIF-dependent state and regained their capacity to participate in the formation of chimeric mice. Molecularly, LIF directly controls *Pramel7* expression, involving both STAT3-dependent transcriptional regulation and PI3K-dependent phosphorylation of glycogen synthase kinase 3β (GSK3β). *Pramel7* expression in turn confers constitutive self-renewal and prevents differentiation through inactivation of extracellular signal-regulated kinase phosphorylation. Accordingly, knockdown of Pramel7 promotes ESC differentiation in presence of LIF and even on forced STAT3-activation. Thus, with this study we demonstrated that Pramel7 represents a central and essential factor in the signaling network regulating pluripotency and self-renewal in ESCs.

Rats represent an optimal animal model for the investigation of human diseases and in some research fields is often, due to its bigger size and higher genetic diversity, preferred to the mouse. Despite the benefits of working with rats, the impossibility of generating germ line competent rat ESCs has given the mouse a clear advantage over the rat in the generation of new transgenic models. All the efforts made for establishing authentic rat ESCs have failed in the last 30 years and only recently, by using defined culture conditions, genuine pluripotent rat ESCs have been established for the first time, from different strains. Although, both mouse and rat ESCs can be derived from the ICM cells, they differ in their *in vitro* stability.
We aimed in our study at the identification of differences at the transcriptional level between the mouse and the rat during the developmental period in which the ICM cells are formed, since they represent the source of ESCs derivation. We performed a microarray analysis in which we compared

the transcriptome of mouse and rat morula, blastocyst, and ICM. This is the first study investigating the gene expression changes during the transition from morula to blastocyst in the rat preimplantation development. Moreover, it represents a new example of statistical approach for cross species analysis, applicable also to other species comparisons, that allows to highlight the species-specific behaviour of genes within important pathways and families. In order to identify alternative regulation of important molecular mechanisms the investigation of differential gene expression between the two species was extended at the level of signaling pathways, gene families, and single selected genes of interest. This study reports for the first time that in the pluripotent pool of cells of the rat and mouse preimplantation embryo substantial differential regulation of genes is present, which might explain the difficulties observed for the derivation and culture of rat ESCs using murine conditions. Some of the genes differentially expressed are already known to be important factors in the maintenance of pluripotency in ESCs, like for example *Sox2* or *Stat3*, or play a role in reprogramming somatic cells to pluripotency like *c-Myc*, *Klf4* and *p53* and would therefore represent interesting candidates to further analyze *in vitro* in the rat ESCs.

The differential regulation of critical genes could represent the starting point for analyzing their function *in vitro* in mouse and rat ESCs. Furthermore, this knowledge could be critical for the improvement of the derivation and maintenance of rat ESCs. A broader knowledge on the molecular mechanisms that occur in rat ESCs would improve the efficiency of establishing stable authentic pluripotent rat ESCs and therefore it would facilitate the generation via gene targeting of transgenic rat models, which are indispensable for the biomedical research.

ZUSAMMENFASSUNG

Das erste Differenzierungsereignis während der Embryonalentwicklung in der Präimplantationsphase ist der Übergang vom Morula- zum Blastozystenstadium.
Dabei durchleben die Morulazellen die allererste zelluläre Spezifizierung, welche in zwei voneinander unterscheidbaren Zellpopulationen resultiert, dem Trophoblasten und der inneren Zellmasse (ICM). Es wird angenommen, dass embryonale Stammzellen (ES-Zellen) mit ihrer Fähigkeit, sich unendlich erneuern zu können, das *in vivo*-Pendant zu den Zellen der inneren Zellmasse darstellen.
Die Kultivierung und Proliferation von embryonalen Stammzellen *in vitro* wird durch einen einzigartigen und komplexen Signalverbund ermöglicht. Dieser hält die Zellen in einem pluripotenten Zustand, ohne dass diese die Eigenschaft verlieren, Gewebe aller drei Keimblätter bilden zu können.
Dieser Zustand kann nur erreicht werden, wenn einerseits der Selbsterneuerungsmechanismus funktioniert und andererseits die Differenzierung in genügendem Masse unterdrückt wird, was bedeutet, dass eine bestimmte Balance zwischen Selbsterneuerung und Differenzierung gewährleistet sein muss. In den letzen Jahrzehnten wurde ein enormer Aufwand betrieben, um dem molekularen Geheimnis der Pluripotenzerhaltung *in vitro* und *in vivo* auf den Grund zu kommen. Trotzdem ist immer noch sehr unklar, wie die Mechanismen genau funktionieren.
Das Gesamtziel dieser Studie war es deshalb, weiteres Wissen über die molekularen Prozesse, welche die Pluripotenzerhaltung kontrollieren, zu erlangen. Die Arbeit wurde anfänglich in zwei verschiedene Studien aufgeteilt: Die erste befasste sich mit der Funktion von Genen, welche mit Pluripotenz in Verbindung gebracht werden können, die zweite bestand in einer vergleichenden Genexpressionsanalyse von Maus- und Rattenembryonen während verschiedenen Präimplantationsstadien.

Es ist bekannt, dass die Aktivierung des LIF/STAT3 Signalwegs ausreicht, um ES-Zellen in einem undifferenzierten und pluripotenten Zustand zu erhalten. Deshalb untersuchten wir in einem ersten Ansatz die Wichtigkeit von STAT3 in der Etablierung von ES-Zellen. Um neue STAT3-Zielgene zu identifizieren, wurden STAT3-überexprimierende ES-Zellen aus einem nicht-permissiven Hintergrund etabliert, um später deren Expressionsprofil mit demjenigen von Wildtyp ES-Zellen zu vergleichen. Mehrere Gene zeigten eine veränderte Expression. Einige von ihnen wurden als potentielle Schlüsselfaktoren der Pluripotenzerhaltung in Betracht gezogen. Von *Pramel6* und *Pramel7,* zwei dieser Kandidatengene, wurde die Funktion umfassend analysiert. Dabei stellte sich heraus, dass *Pramel7* (preferentially expressed antigen in melanoma-like 7) eine wichtige Rolle in der Erhaltung der Pluripotenz und in der vom LIF/STAT3 Signalweg vermittelten Selbsterneuerung von ES-Zellen einnimmt. Es zeigte sich weiter, dass *Pramel7 in vivo* exklusiv in vollumfänglich pluripotenten Zellen exprimiert wird, also im zentralen Zellkompartement des Morulastadiums und in der inneren Zellmasse der Blastozyste. *In vitro* wurde beobachtet, dass das Fehlen von *Pramel7*-Expression zur Differenzierung von ES-Zellen führt, dessen Überexpression hingegen zur Folge hatte, dass die Selbsterneuerung der Zellen ohne Zugabe von exogenem LIF über längere Zeit aufrecht erhalten werden konnte. Darüber hinaus unterdrückte die Überexpression von *Pramel7* die Differenzierung von ES-Zellen *in vitro* und *in vivo*. Dieser Prozess ist umkehrbar: Nach der Entfernung des *Pramel7*-Transgens, welches die Überexpression verursachte, wurden die Zellen erneut LIF-abhängig und erlangten die Fähigkeit, Chimären zu bilden. Auf molekularer Ebene wird *Pramel7* direkt durch LIF kontrolliert, einerseits via STAT3-abhängige Transkriptionsregulation, andererseits durch PI3K-abhängige Phosphorylierung der Glycogen Synthase Kinase 3β (GSK3β). *Pramel7*-Expression wiederum gewährleistet konstitutive Selbsterneuerung und verhindert Differenzierung durch Inhibition der Phosphorylierung der extracellular signal-regulated kinase (ERK). Dementsprechend begünstigt der Knockdown von *Pramel7* die Differenzierung von ES-Zellen, auch unter Zugabe von exogenem LIF und sogar nach erzwungener STAT3-Expression. Zusammenfassend wurde in dieser Studie gezeigt, dass *Pramel7* eine zentrale und wichtige Rolle im pluripotenzerhaltenden Signalverbund der Selbsterneuerung von ES-Zellen darstellt.

Die Ratte ist ein optimales Modell für die Untersuchung von menschlichen Krankheiten. In einigen wissenschaftlichen Bereichen wird sie der Maus vorgezogen, hauptsächlich wegen dem grösseren Körper und der höheren genetischen Diversität. Trotz dieser Vorteile war es für lange Zeit nicht möglich, keimbahnkompetente ES-Zellen von Ratten zu generieren, was der Maus im Bereich der Herstellung von transgenen Modellen einen klaren Vorteil verschaffte. In den letzten 30 Jahren schlugen trotz grösster Anstrengungen alle Versuche fehl, authentische Ratten ES-Zellen zu generieren. Erst kürzlich gelang es einer Forschungsgruppe funktionelle ES-Zellen von verschiedenen Rattenstämmen unter definierten Kulturbedingungen zu derivieren. Obwohl nun ES-Zellen aus der inneren Zellmasse von Maus und Ratte hergestellt werden können, unterscheiden sich die Zelllinien der verschiedenen Spezies in ihrer Stabilität *in vitro*.

Die zweite Studie setzte sich zum Ziel, Unterschiede zwischen Maus und Ratte während der Transition vom Morula- zum Blastozystenstadium auf transkriptioneller Ebene aufzudecken, da die Zellen der inneren Zellmasse der Blastozysten das Ursprungsmaterial für die Generierung von ES-Zellen darstellen. Dafür wurde eine Microarrayanalyse durchgeführt, in welcher die Transkriptome von Morulae, Blastozysten und innerer Zellmassen der Maus und der Ratte verglichen wurden. Dies ist die erste Studie, welche die Veränderungen der Genexpression während dem Übergang vom Morula- zum Blastozystenstadium untersucht. Darüber hinaus repräsentiert sie einen neuen statistischen Ansatz für die Expressionsanalyse zwischen zwei verschiedenen Spezies und ist auch auf andere Artenvergleiche anwendbar, was das Bestimmen von artenspezifischem Verhalten von Genen in wichtigen Signalwegen und Genfamilien ermöglicht. Um eine unterschiedliche Regulation von wichtigen molekularen Mechanismen zu identifizieren, wurde die Detektion von sich zwischen den beiden Spezies unterscheidender Genexpression auf das Niveau von Signalwegen, Genfamilien und ausgewählter Einzelgene angelegt. Die Untersuchung zeigt zum ersten Mal, dass sich die Genexpression im pluripotenten Zellpool von Embryonen des Präimplantationsstadiums zwischen Maus und Ratte teilweise substantiell unterscheidet. Dies könnte mit ein Grund sein, weshalb es bis anhin so schwierig war, ES-Zellen der Ratte unter Mausbedingungen zu generieren und zu kultivieren. Einige der Gene, bei welchen zwischen den Arten eine unterschiedliche Expression hat festgestellt werden können, sind schon länger als wichtige Faktoren in der Pluripotenzerhaltung von ES-Zellen bekannt, dazu gehören unter anderem *Sox2* oder *Stat3*, oder sie spielen eine Rolle in der Reprogrammierung von somatischen zu pluripotenten Zellen, wie *c-Myc*, *Klf4* und *p53*. Diese Gene könnten deshalb interessante Kandidaten für weitere *in vitro* Analysen von ES-Zellen der Ratte sein.
Diese unterschiedliche Regulierung von kritischen Genen könnte ein Auslöser für die Untersuchung von deren Funktion *in vitro* an ES-Zellen von Maus und Ratte sein. Des Weiteren könnte dieses Wissen zu Verbesserungen in der Etablierung von ES-Zellen von Maus und Ratte führen. Dafür bedarf es auch in Zukunft eines umfangreicheren Verständnisses der molekularen Mechanismen, welche für die Kontrolle und Erhaltung von Pluripotenz verantwortlich sind. Dies, um authentische und pluripotente ES-Zellen der Ratte zu derivieren und somit auch die Herstellung von transgenen Tieren via Gentargeting zu erleichtern, denn solche Modelle sind in der biomedizinischen Forschung unentbehrlich.

OUTLINE

This thesis comprises several parts, which I would like to outline here.

The introduction provides basic information about embryonic stem cells (ESCs) and their applications. After the first chapter focusing on the derivation and maintenance of murine ESCs in culture, a second chapter follows regarding the rat as an animal model for the research. Furthermore, preliminary study are mentioned where rat ESCs have been established for the first time. In the last part of the introduction a general overview of the molecular signature of ESCs is reported. This part of the introduction is a shorter version of a work written for the book "Embryonic Stem Cells: The Hormonal Regulation of Pluripotency and Embryogenesis", where I wrote the chapter "Molecular mechanisms of pluripotency" (Casanova et al., 2011a). In the last part of the introduction the aims of the work are reported.

The results section consists in the publications obtained during the PhD. This chapter is divided into two main parts, which represent the two projects I was working on. In the first section are reported the papers of two consecutive studies, which aimed at the characterization of newly identified potential STAT3 target genes in murine ESCs. In the last part the results of my second project in the form of a manuscript that as recently been submitted are reported. In this study we analyzed the whole genome expression in the mouse and in the rat preimplantation embryos, with the intention of highlighting differences in the gene regulation in the two species for an improvement of the ESCs derivation.
The three papers are listed at the end of the thesis as Annex.

The discussion section follows the structure used in the results part, being divided into two main chapters that represent the two projects of my PhD. Regarding the first project, the relevant findings obtained from the study on *Pramel7* gene are discussed and follow-up studies are described. Additionally, there is a short discussion about the gene *Pem/Rhox5*, which we detected differentially regulated upon STAT3 overexpression, but we did not so far further characterize its function in the context of maintenance of pluripotency *in vivo* as well as *in vitro*.
Concerning my second project on mouse and rat cross-species genome wide expression analysis in the preimplantation embryos, are initially discussed the technical problems we had to overcome for the collection of all the embryos needed for the microarray analysis. Thereafter, the data obtained from the cross-species comparison are briefly discussed and put in the context relative to ESCs. Seen that this study is the first approach aimed at the identification of differential gene regulation in the two species, this paragraph ends with some remarks about follow-up studies which intend identify from our analysis genes involved in the maintenance of pluripotency in rat ESCs.
The outlook, finally, proposes experimental approaches for a further characterization of the molecular mechanisms controlling pluripotency in ESCs, on the basis of our findings in both the projects performed during my PhD.

A. INTRODUCTION

Embryonic stem cells (ESCs) are pluripotent cells, which can be isolated from the inner cell mass (ICM) of blastocyst stage embryos. They are defined by two properties: they can indefinitely self-renew *in vitro* and they can contribute to the formation of all cells of an adult organism, including functional gametes for genome transmission. Due to their pluripotent state ESCs can be used for various applications, like the generation of knockout or transgenic animals, and potentially as a cell source for cell therapy in regenerative medicine. Alternatively self-renewing cells with pluripotent potential can also be generated by specifying germ cells with extrinsic factors (Matsui, 1992) or by reprogramming somatic cells using gene transfection to generate the so called induced pluripotent stem cells (iPSCs) (Nakagawa et al., 2008; Takahashi and Yamanaka, 2006).
Due to the fundamental characteristics of ESCs, developmental biology, regenerative medicine and cancer biology are more and more interested in understanding the molecular mechanisms controlling stem cells. Even though a lot of efforts have been made in the past years to elucidate the factors that regulate stem cell self-renewal and pluripotency, the precise mechanism of how these processes are regulated remains largely unknown. For a reliable establishment of ESC cultures *in vitro*, the characterization of further marker genes is of fundamental importance. Therefore one of the aims of this PhD thesis was to functionally characterize *Pramel7*, a newly identified pluripotency-related gene (Cinelli et al., 2008). *In vivo* pluripotent cells do exist only transiently during the embryo preimplantation development; therefore an analysis of the naturally existing pluripotent cells could be of advantage for a better understanding of the molecular mechanisms that occur *in vitro*, in the ESCs. For this reason the second intent of this work was to compare in a genome-wide study the pluripotency-related gene networks in the mouse and in the rat morula and blastocyst stage embryos, by performing an analysis of the transcriptome. The purpose of this study was to identify differences and similarities in the molecular processes that occur in the two species in the two analyzed developmental stages. These findings could in the future help in the identification of important genes involved in the maintenance of pluripotency in rat ESCs.

1. Pluripotent embryonic stem cell

Stem cells can be found in embryos, fetuses, or in adults, and by definition, they have the ability to reproduce themselves for a long period of time while keeping the capability to differentiate into diverse cell types. Adult stem cells are undifferentiated cells, found throughout the body after embryonic development, that multiply by cell division to replenish dying cells and regenerate damaged tissues. Pluripotent embryonic stem cells have broader potential since they are able to generate all types of cells that form the three germ layers (mesoderm, endoderm, and ectoderm) from which all the cells of the body arise. Besides ESCs, two other type of cells have been derived from the early mouse embryo that can be regarded as stem cells: the trophoblast stem cells, and the extraembryonic endoderm cells (Martin, 1981; Tanaka et al., 1998). Nevertheless immortal pluripotent cell lines with self-renewing capacity can exclusively be obtained by culturing ICM cells of the blastocyst (Fig. 1).

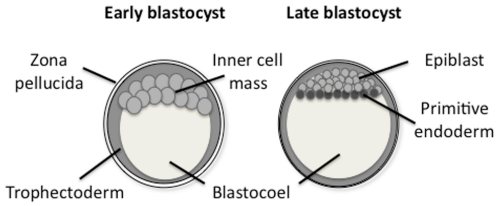


Fig. 1: Development of mouse blastocyst. The outer layer of the late morula forms an epithelium called the trophectoderm. Around embryonic day E3, the blastocoel forms and moves the inner cell mass to one end of the blastocyst. During late blastocyst stage, just prior to hatching from the zona pellucida, cells of the inner cell mass become organized into epiblast and primitive endoderm populations. The isolated ICM give rise to the pluripotent ESCs once cultivated under the appropriate culture conditions.

1.1 Historical overview

During the seventies it was observed that transferring 1 to 7.5 days old mouse embryos to the extra-uterine site in a histocompatible host, induces the generation of teratocarcinomas. These tumors contain stem cells called embryonal carcinoma cells (ECCs), which can be isolated and cultivated *in vitro*. Due to their ability to differentiate into diverse cell types and to participate to the development of normal adult mice once injected into blastocysts, they have been extensively used as a model system for developmental studies. These findings raised the question about the origin of these pluripotent cells and if there was the possibility to generate pluripotent cell lines directly from the embryo and not from a teratocarcinoma. Few years later two laboratories independently answered this question.

In the year 1981, M. J. Evans and M. H. Kaufman established for the first time *in vitro* progressively growing cultures of embryo-derived cells by cultivating mouse blastocyst embryos (Evans and Kaufman, 1981). The transient existence of a population of pluripotent cells during this developmental stage was already known, nevertheless all the attempts to obtain stable cultures of pluripotent cells have been unsuccessful. Comparison between cell surface antigens and protein patterns synthesis with the established culture of ECCs showed high homology with the epiblast cells of the early postimplantation mouse embryo. Due to the difficulties to isolate early implanted embryos and because of the small amount of cells forming the epiblast population, Evans and Kaufman used diapause-induced blastocyst. This is a particular phenomenon that has evolved in certain mammals to overcome sub-optimal conditions for pregnancy. Thus, mice can temporary arrest embryogenesis just before implantation at the blastocyst stage. This condition is induced by maternal hormones, but can be artificially provoked by ovariectomy and hormonal treatment. Even though during this time, which in the mouse can persist for weeks, no further development of the blastocysts occurs there is a gradual increase in the cell number. Taking advantage of this phenomenon, Evans and Kaufman could isolate from the uterus of ovariectomized mouse females 4 to 6 days delayed blastocysts (Evans and Kaufman, 1981). After 4 days in culture the trophectoderm cells of the blastocysts developed into giant trophoblast cells whereas the ICM cells formed round, compact structures. Once transferred on dishes containing a layer of inactivated fibroblasts (referred as to feeders), the ICM-derived cells started actively to proliferate and formed colonies that resembled the ones generated by ECCs. Importantly, a typical characteristic of ECCs is an unstable karyotype and usually they do not contain the Y chromosome. Analysis of the ICM-derived cells showed a stable diploid karyotype and both XX and XY cell lines were established and maintained in culture for several passages. The authors termed these embryo-derived cells EK cells to distinguish them from the tumor-derived EC cells. The pluripotent potential of the EK cells was confirmed by subcutaneously injection into mouse flanks and subsequent formation of tumors that contained derivatives of all the three germ layers. When cultivated in the absence of feeders, the same cells formed embryoid bodies that differentiated into specialized cell types (Evans and Kaufman, 1981).

In the same year also in a second laboratory embryo-derived pluripotent cell lines were established for

the first time; Martin G. R. called these cells embryonic stem cells (ESCs) (Fig. 2) (Martin, 1981).

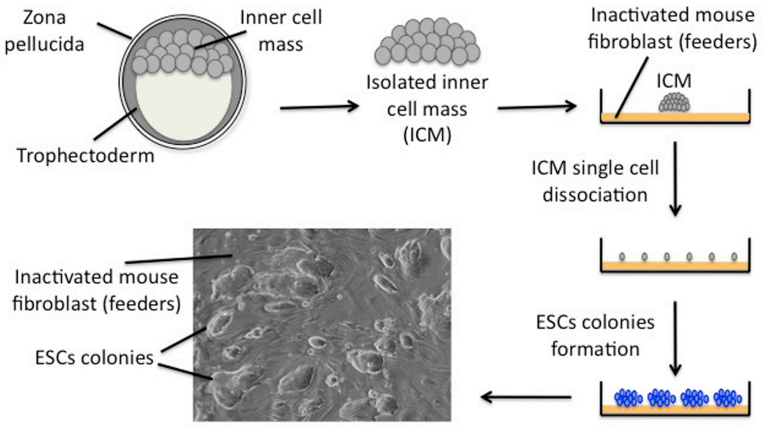


Fig. 2: Establishment of ESCs culture. The ICMs of blastocyst stage embryo are isolated and cultivated for few days on a layer of mitotically inactivated fibroblast cells (feeders). ICMs are then dissociated into single cells, which are plated into a new culture dish prepared with feeders. Single cells grow and form colonies of proliferating ESCs.

It was believed that the ability of ECCs to proliferate was due to the production of autocrine factors that promoted growth and/or blocked differentiation. Therefore with this assumption, Martin cultivated blastocyst stage embryos in an ECC-conditioned medium (Martin, 1981). The isolated blastocysts were subjected to immunosurgery treatment, which allow the isolation of the ICM cells by selectively killing the trophoblast cells (Solter and Knowles, 1975). The obtained ICMs were then cultivated on a layer of feeders with the ECC-conditioned medium. After one week these cells formed many ECC-like colonies and were expanded for several passages without the need of conditioned medium, confirming the idea that proliferating cells endogenously express factors required for maintaining their characteristics. This was not the case for the ICMs cultivated without the conditioned medium, which very early stopped to proliferate. Different lines of ESCs were established using this protocol and all of them retained the ability to differentiate *in vitro* as well as *in vivo*. Martin hypothesized the existence of a cell-autonomous production of a factor able to sustain ESCs proliferation. Nevertheless, because ICMs cultivated in absence of ECC-conditioned medium did not generate ESCs, the possibility that the feeders produced this proliferation-promoting factor was excluded (Martin, 1981). Not many years later it was demonstrated by other laboratories that the use of diapause-blastocysts or of ECC-conditioned medium was dispensable for a successful generation of pluripotent ESCs (Axelrod, 1984).
In 1988 A. Smith and colleagues isolated a soluble glycoprotein that prevents stem cell differentiation and established that ESC self-renewal is dependent on paracrine signals produced from the feeders on which ESCs are cultivated (Smith et al., 1988). The principal factor required for self-renewal was shown to be leukemia inhibitory factor (LIF) (Gearing et al., 1987; Williams et al., 1988). *Lif* knockout feeders were reported to be unable to support ESCs self-renewal (Stewart et al., 1992), indicating that supply of LIF was a key attribute of feeders.

1.2 Derivation and maintenance of embryonic stem cells

Derivation and maintenance of murine ESCs *in vitro* was originally achieved by using feeders and/or LIF in combination with fetal calf serum and/or the growth factor bone morphogenetic protein (BMP) (Smith et al., 1988; Ying and Smith, 2003). However, the same culture conditions are not sufficient for derivation of ESCs from most of the mouse strains and not at all from the rat. The genetic background strongly affects the efficiency of ESC isolation. Even though ESCs were discovered more than 25

years ago only limited number of ESCs of proven ability to colonize the germ-line have been obtained and only a few mouse strains other than 129 (Simpson et al., 1997). Only after adjusting the culture conditions, germ line competent inbred ESCs could be established, e.g. from C57BL6/J (Keskintepe et al., 2007; Ledermann and Bürki, 1991), DBA/1lacJ (Roach et al., 1995), BALB/c (Kawase et al., 1994; Noben-Trauth et al., 1996), and CBA mice (Lodge et al., 2005). Recently it has been shown that extrinsic stimuli (like LIF, serum, and feeders) are dispensable for derivation and maintenance of the pluripotent state. Culture conditions free from feeders, serum and cytokines were established by using a combination of small-chemical molecules, which inhibit the fibroblast growth factor (FGF)/mitogen-activated protein kinase (MEK)/extracellular signal-related kinase (ERK1/2) and the glycogen synthase kinase 3 (GSK3) (Ying et al., 2008). These culture conditions are known as 3i or 2i and have been applied for derivation of ESCs from non-permissive mouse strains like non-obese diabetic (NOD) mice (Nichols et al., 2009a). Nevertheless in absence of LIF or 2i, murine ESCs exit self-renewal and differentiate into different cell types, demonstrating that maintenance of the undifferentiated stem cell phenotype is not completely cell-autonomous (Fig. 3).

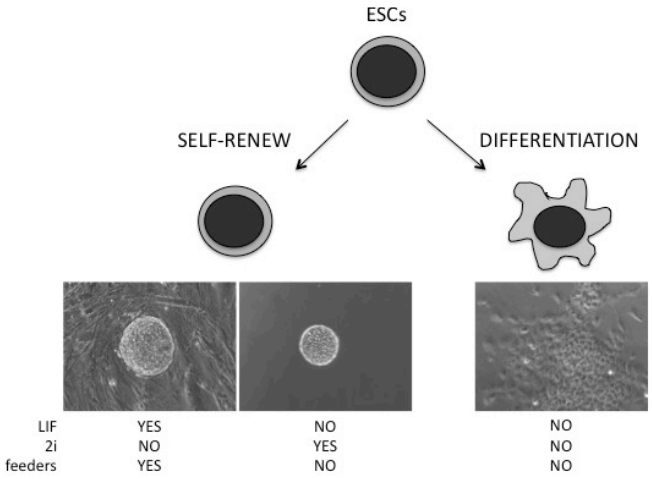


Fig. 3: ESCs self-renewal and differentiation. Undifferentiated ESCs have small size and have a high nuclear to cytoplasmic ratio (left), which is lost once the cells differentiate (right). Undifferentiated ESCs form round shape and compact colonies that can be indefinitely expanded, either in presence of LIF and feeders or only 2i. On the other hand in absence of these factors ESCs differentiate and form monolayer colonies, which lose the compact, round shape typical for undifferentiated cells.

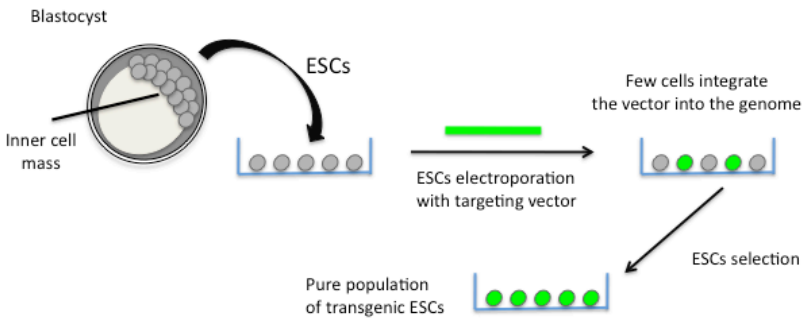
On the molecular level, pluripotent ESCs are characterized by the expression of specific cell surface glycoproteins such as the stage-specific embryonic antigen 1 (SSEA-1) (Solter and Knowles, 1978) as well as by the presence of transcription factors such as OCT3/4 (Schöler, 1991; Schöler et al., 1989) and Nanog (Chambers et al., 2003). High expression levels of alkaline phosphatase also characterize ESCs. Furthermore, ESCs exhibit a short G1 phase of the cell cycle (Rohwedel et al., 1996) and a high telomerase activity (Thomson et al., 1998). Other essential properties of ESCs include growth as multicellular colonies, normal and stable karyotypes, and prolonged undifferentiated culture.

1.3 Manipulation of ESCs genome: a powerful tool

Pluripotent cells, which can differentiate into all types of cells *in vivo* as well as *in vitro*, represent an important source from which we can learn and study different molecular processes. ESCs with their wide differentiation potential are useful tools for studying developmental processes that take place *in vivo* in the embryo. The developmental potency of murine ESC is routinely tested with three different assays: first, *in vitro* by giving the right extrinsic signals ESCs can be differentiated into a variety of cell types; second, ESCs once injected into immunosuppressed mice forms teratomas that are composted from cells of all the three germ layers; third, ESCs injected into a host blastocyst take part to the development of the embryo.

For understanding the function of one gene, loss of function and gain of function mutations are necessary. While gain of function mutations are routinely produced in mammals including mouse and rat by injecting DNA into one of the pronuclei of the fertilized egg, targeted loss of function (knockout) mutations can routinely only be generated for the species where ESCs are available. Therefore, due to their properties, one of the biggest interests of ESCs has been their utility as cellular vectors for engineering the genome (Fig. 4A). With gene targeting, via transfection with modified homologous DNA, the genome of ESCs can be manipulated. Once the transgenic ESCs are returned to the embryo microenvironment they can participate in its development and produce viable chimeras (Fig. 4B). This technique of gene targeting via ESCs is one of the most powerful tools to investigate gene regulation and function *in vivo* and for modeling human genetic disorders and diseases. Since the establishment of murine germline competent ESCs the mouse is by far the most studied model organism.

A



B

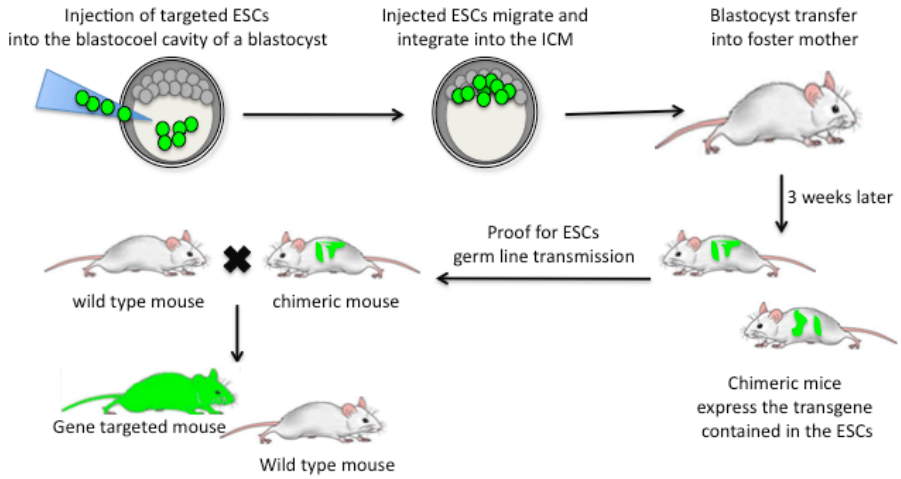


Fig. 4: **A.** Gene targeting in ESCs. ICM isolated ESCs are genetically modified by electroporation with a vector containing the desired transgene (shown in green). Thereafter ESCs are treated with selection media for enriching the population carrying the modified gene. **B.** Gene targeted mouse. The transgenic ESCs (green dots) are injected into the blastocoel of host blastocyst and after few hours ESCs migrate into the ICM. Injected embryos are then transferred to pseudo-pregnant female. Transgenic ESCs participate to the embryo development generating chimeric mice. Mating chimeric mouse with wild type mouse proves germ line transmission of the mutation.

2. The rat: an important animal model

Rats represent an optimal animal model for the investigation of human diseases and in some research fields is often, due to its bigger size and higher genetic diversity, preferred to the mouse. Due to its bigger size compared to the one of the mouse, interventions such as organ transplantation or *in vivo* functional analyses such as blood pressure measurements are easier to perform. Moreover, the higher genetic diversity allows the overcoming of species specific alterations (Canzian, 1997). Therefore, rats are preferred for instance, for the study of neurodegenerative diseases and disorders affecting higher brain function, such as depression and addiction, or for studies of transplantation biology, toxicology and drug development.
Despite the benefits of working with rats, the impossibility of generating germ line competent rat ESCs has given the mouse a clear advantage over the rat in the generation of new transgenic models. With a lot of efforts transgenic rats have been generated by using alternative techniques, like random mutagenesis with ENU (N-ethyl-N-nitrosourea), gene knockdown with RNA interference, or genome modification of somatic cells combined with nuclear-transfer (Hasuwa et al., 2002; Zan et al., 2003; Zhou et al., 2003). Nevertheless, all these approaches have major limitations compared to the gene targeting procedure via ESCs used for generating transgenic mice. For example, for generating the first two knockout rats using the random mutagenesis ENU, about 2000 pups have been screened before the mutation was found in the gene of interest (Zan et al., 2003). ENU induces heritable genetic changes and was estimated to functionally mutate one allele every 1000 alleles analyzed (Justice et al., 1999; Noveroske et al., 2000). One major problem of using this technique is that the mutations are randomly generated into the genome, therefore a large number of animals have to be generated before a mutation hits the selected gene. Moreover, even if the selected gene is mutated and functionally not active anymore, ENU causes always more than one single mutation in the genome. Therefore, in ENU-treated animals it is not to exclude that the phenotype seen is directly caused by the mutation in the gene of interest but rather to an accumulation of random mutations. However, the first two knockout rats for the breast cancer suppressor genes *Brca1* and *Brca2* showed different phenotypes than a similar model in the mouse (Zan et al., 2003). Interestingly, in the mouse functional mutation in *Brca*2 gene has lethal effect on the embryo (Friedman et al., 1998; Suzuki et al., 1997). In the rat, in contrast no lethal phenotype was observed but only growth defects and gonadal atrophy (Zan et al., 2003). Thus, these divergent results demonstrate how the species influence the final phenotype of the mutation and illustrate the importance of having different animals at disposal to use as models for investigating human diseases.

2.1 Rat embryonic stem cells

Despite the big potential of ESCs and the advantages that working with rat as disease model offers, all the efforts made for establishing authentic rat ESCs have failed in the last 30 years (Iannaccone et al., 1994; Ruhnke et al., 2003; Vassilieva et al., 2000).
Only recently, by using molecular defined culture conditions (2i conditions: Ying et al., 2008), for the first time genuine pluripotent rat ESCs have been established from different strains (Buehr et al., 2008; Li et al., 2008).
Interestingly, the presence of serum in the medium induced fast differentiation of rat ESCs, even in presence of LIF (Li et al., 2008). Interestingly, murine ESCs can be established and cultivated under 2i conditions in absence of LIF and feeders. However, this is not the case for rat ESCs, which need a feeder layer and besides the presence of 2i they are also cultivated in LIF containing medium (Buehr et al., 2008). These differences might explain why all attempts to derive rat ESCs, by using conditions developed for mouse ESCs culture, have failed in the last 30 years.

In sum, finding the right culture conditions allows the establishment of authentic ESCs apparently without strain and species limitations, at least for the mouse and the rat. Nevertheless, the most crucial point remains the understanding of the molecular mechanisms that govern ESCs pluripotency, seen that ESCs and the induced pluripotent stem cells (iPSCs) hold great promise for the therapeutic treatment of human diseases.

3. The molecular signature of ESCs

The unique characteristics of ESCs are due to the particular molecular signature that these cells exhibit. The mystery of pluripotency will be unravelled once the interplay between transcription factors, pathways and the epigenetic machinery will be understood. The following paragraphs present a review of the most important molecular and cellular mechanisms that regulate stem cell self-renewal and pluripotency prevalently in mouse ESCs, since they are the most investigated ESCs.

3.1 The core transcription factors: OCT3/4, SOX2, and Nanog

A critical role in maintaining ESC identity is played by a set of transcription factors centred on the OCT3/4 (Nichols et al., 1998; Schöler, 1991), the SRY-related HMG-box gene 2 (SOX2) (Yuan et al., 1995), and Nanog (Chambers et al., 2003; Mitsui et al., 2003). Genome-wide studies have highlighted the co-localization of these three transcription factors in ESCs chromatin, increasing the complexity of the transcriptional networks that direct ESC identity.

3.1.1 The transcription factor OCT3/4

OCT3/4 is a member of the POU transcription factor family; it recognizes an 8-base pairs DNA sequence found in the promoters and enhancer regions of many ubiquitously expressed and cell-specific genes (Ruvkun and Finney, 1991). The gene encoding OCT3/4 is named *Pou5f1*. OCT3/4 was first identified as an active binding factor in the extract of undifferentiated embryonic stem and embryonal carcinoma cells (Lenardo et al., 1989; Schöler, 1991). The presence of OCT3/4 protein in ESCs and embryonal carcinoma cells first suggested an association with the early stage of mouse embryogenesis. During the mouse development, it is first detected in oocytes and its expression declines during the first two cleavages divisions, but it reappears at the 4-8 cell-stage, where it is expressed in all the nuclei. Subsequently it is reduced in the trophectoderm and becomes restricted to the ICM. In the post-implantation embryos, OCT3/4 is localized in the epiblast but disappears as cells undergo differentiation, with expression persisting in the germ cells (Palmieri et al., 1994). The importance of OCT3/4 during early embryogenesis was also highlighted by the fact that embryos lacking the *Pou5f1* gene die after implantation due to the absence of the ICM (Nichols et al., 1998). The requirement of OCT3/4 in the maintenance of developmental potency was determined by using a conditional expression and repression system in ESCs (Niwa et al., 2000). A critical amount of OCT3/4 is needed to sustain self-renewal, and up- or downregulation induces differentiation of the ESCs. Overexpression of this factor promotes differentiation into primitive endoderm and mesoderm, whereas repression of it causes loss of pluripotency and differentiation into trophectoderm (Fig. 5). Effectively, *in vivo* OCT3/4 is abundant in the ICM cells of the blastocyst and down-regulated in the trophectoderm, whereas in the primitive endoderm the level of expression increases (Palmieri et al., 1994). These findings highlight the fundamental function of OCT3/4 in preventing dedifferentiation of epiblast cells or of ESCs into trophectoderm lineage. The sole exogenous expression of OCT3/4 is sufficient to generate pluripotent stem cells from mouse neural stem cells (Kim et al., 2009) and together with three other transcription factors it is essential in the reprogramming of somatic cells into the pluripotent state (Nakagawa et al., 2008; Takahashi and Yamanaka, 2006). While the other factors involved in reprogramming are replaceable by family members or other factors, without OCT3/4 no reprogramming occurs (Nakagawa et al., 2008). Moreover knockdown experiments in ESCs showed a very dramatic change in gene expression compared to the one caused for example by Nanog or SOX2 knockdown (Ivanova et al., 2006) implying OCT3/4 as a chief selector for ESC fate decision.
Of the known OCT3/4 target genes, four have been shown to be essential for the maintenance of pluripotency: *Sox2*, the undifferentiated transcription factor 1 (*Utf1*), *Rex1/Zfp42* and *Nanog*. The regulatory regions of these genes contain an octamer element capable of binding OCT3/4. As we will describe in the following paragraphs, *Sox2* is not only an OCT3/4 target gene but also serves as a cofactor for OCT3/4 (Okumura-Nakanishi et al., 2005). Although OCT3/4 and SOX2 have independent roles in determining other cell types, in pluripotent cells they act synergistically to drive

transcription of their target genes. Furthermore, this complex was found to promote transcription of the Nanog homeoprotein (Rodda et al., 2005) (Fig. 5).

3.1.2 The transcription factor SOX2

SOX2 is a member of the sex-determining region of the Y chromosome-related (SRY-related) high-mobility group (HMG) box (SOX) family of transcription factors. SOX2 has an expression pattern similar to that of OCT3/4 through the mouse preimplantation development. SOX2 expression is also associated with precursor cells of the developing central nervous system and indeed can be used to isolate these cells. Like OCT3/4 downregulation of SOX2 correlates with a commitment to differentiation, and is no longer expressed in cell types with restricted developmental potential. In contrast to OCT3/4, the phenotypic lethal consequences of the absence of SOX2 expression in the embryo are visible only after implantation (Avilion et al., 2003). This is due to an accumulation of maternal SOX2 in the cytoplasm of the oocytes, which persist in all cells at least until the blastocyst stage, and not like OCT3/4 maternal transcripts, which last only till the 2-cell stage embryo (Palmieri et al., 1994). Thus, mutant embryos lacking the *Sox2* gene die presumably when the maternal SOX2 becomes diluted causing the differentiation of the epiblast cells into trophectoderm or extraembryonic ectoderm (Avilion et al., 2003) (Fig. 5). As mentioned before, loss of OCT3/4 causes differentiation of the epiblast cells into trophectoderm derivatives (Niwa et al., 2000). Avilion et al. proposed that the presence of both transcription factors is required for the formation and maintenance of epiblast cells. An upregulation of OCT3/4 and the accompanying downregulation of SOX2 lead to differentiation towards extraembryonic ectoderm.
The regulatory regions of the *Sox2* gene contain an octamer element capable of binding OCT3/4 (Tomioka et al., 2002; Yuan et al., 1995). A possible interaction of these two factors was also proposed by the fact that almost all the SOX2-OCT3/4 target genes have both the octamer and sox heptamer elements separated by either 0 or 3 base pairs (Reményi et al., 2003; Williams et al., 2004). The *Pou5f1* gene has different regulatory regions that are important for its expression. The distal enhancer, which contains the conserved region CR4, was shown to be required for ESC-specific OCT3/4 expression (Nordhoff et al., 2001). Two regulatory regions (the SRR1 and SRR2), known to confer ESC-specific expression, were also found in the *Sox2* sequence. Using a chromatin immunoprecipitation (ChIP) assay Chew et al. first demonstrated that SOX2 and OCT3/4 interact with the enhancers of *Pou5f1* and *Sox2* genes. Moreover OCT3/4 and SOX2 knockdown experiments showed a reduced enhancer activity, confirming that both factors positively control their reciprocal expression (Chew et al., 2005). The same authors hypothesized a transcriptional regulatory network consisting of auto-regulatory and multi- component loops. In an auto-regulatory system the gene product binds to its own regulatory element allowing its continued and stable expression. In a multi-component system, the OCT3/4 factor binds to the *Sox2* regulatory element and *vice versa* generating a bi-stable system with the possibility to switch between the two different states (Chew et al., 2005) (Fig. 5).
We described before that *Sox2* null embryo die after implantation, because of differentiation of the epiblast cells. The same findings are reproducible also *in vitro*, where ESCs lacking *Sox2* gene differentiate primarily into trophectoderm-like cells. Thus, SOX2 was defined to be indispensable for maintaining ESCs pluripotency. However, the transcription of many OCT3/4-SOX2 target genes was not affected in ESCs null for *Sox2*. These findings suggested that SOX2 regulates the expression of OCT3/4 through the regulation of multiple transcription factors (Masui et al., 2007). So, it seems that the main contribution of SOX2 in ESCs is to maintain OCT3/4 expression. Consistent with this idea is the finding that enforced expression of OCT3/4 can rescue ESCs from differentiation induced by the loss of *Sox2* (Masui et al., 2007). Not to forget is also that SOX2 is one of the four transcription factors, which together are able to induce reprogramming to pluripotency in differentiated cells (Takahashi and Yamanaka, 2006). Furthermore, large-scale ChIP studies have shown the OCT3/4-SOX2 complex closely localized to another important regulator of pluripotency, the Nanog transcription factor.

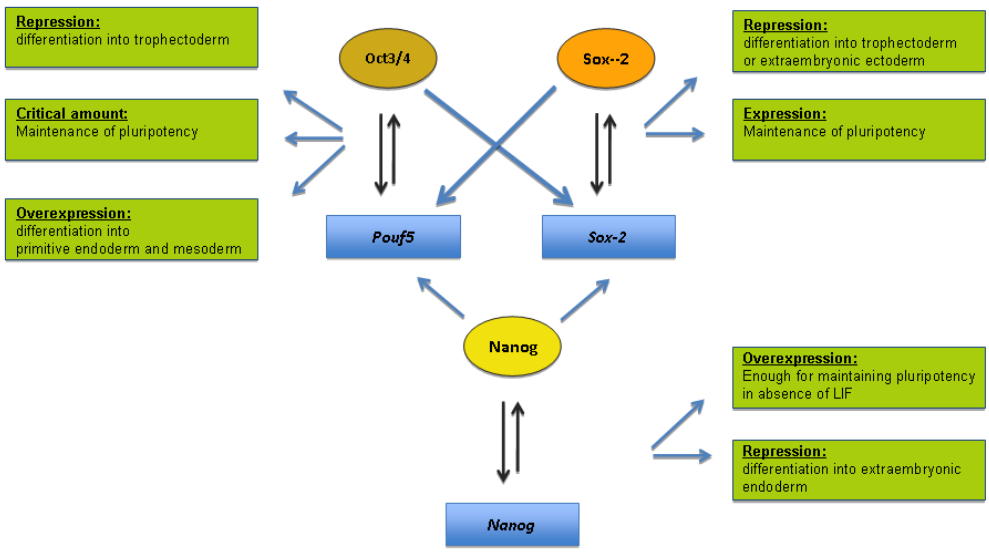


Fig. 5: The "trinity" of nuclear regulators that control stem cell identity: OCT3/4, SOX2, and Nanog transcription factors. Transcriptional regulatory network of the three factors and the effects on cell fate after altering their transcriptional levels. (From Casanova et al., 2011a)

3.1.3 The transcription factor Nanog

Nanog is a homeodomain protein, which acts as an intrinsic effector of ESCs self-renewal. During ESCs differentiation, Nanog mRNA declines markedly, and its expression is retained only in undifferentiated cells. In the mouse embryo its first expression appears in the compacted morula and it is localized to the interior cells, the future ICM. In blastocysts, the expression is confined to the ICM and absent from the trophectoderm. In the later blastocyst, Nanog is further restricted to the epiblast and excluded from the primitive endoderm; it is then down regulated after implantation (Chambers et al., 2003; Mitsui et al., 2003). At day E11.5-E12.5 Nanog expression marks the pluripotent germ cells. Overexpression of Nanog in ESCs was found to allow proliferation of undifferentiated ESCs in absence of LIF (Chambers et al., 2003). After transfection with Cre-recombinase, in which the *Nanog* expression cassette has been eliminated, these cells reverted to LIF-dependence, demonstrating that this phenotype was directly attributable to Nanog overexpression. Following exposure to a differentiation-promoting agent, like retinoic acid, cells overexpressing Nanog remained, in contrast to the Cre-reverted cells, morphologically undifferentiated and expressed OCT3/4. Cre-reverted cells were injected into mouse blastocysts and contributed to the generation of germline competent chimeras. Even though cells overexpressing Nanog self-renew in a cytokine independent manner, the presence of LIF confers to these cells an enhanced self-renewing capacity (Chambers et al., 2003).
A reduction in the level of Nanog causes ESCs to differentiate to extraembryonic endoderm lineages (Chambers et al., 2007; Ivanova et al., 2006) (Fig. 5). *In vivo* the absence of Nanog during embryo development results in early lethality. ICM of *Nanog* null blastocysts failed to proliferate and differentiated into parietal endoderm-like cells, demonstrating that its expression is essential for maintenance of pluripotency of the ICM cells (Mitsui et al., 2003). Interestingly, ESCs upon targeted deletion of the *Nanog* gene can self-renew indefinitely and can contribute to the generation of chimeric animals (Chambers et al., 2007). This indicates that loss of Nanog in ESCs does not affect pluripotency once this was established. Nevertheless, even though *Nanog* null cells colonize the germ layers of the chimeric mice and they are recruited to the germ line, primordial germ cells lacking *Nanog* fail to mature on reaching the genital ridge (Chambers et al., 2007). These data confirm that Nanog is specifically required for the formation of germ cells.
The fact that STAT3 activation in presence of LIF was not increased in cells overexpressing Nanog (Mitsui et al., 2003) and the fact that these cells did not differentiate in presence of a JAK/STAT3 inhibitor (Chambers et al., 2003), clearly demonstrate the existence of a parallel and LIF/STAT3-

independent mechanism sustaining pluripotency in ESCs. *In vivo* these findings are confirmed by the fact that Nanog is absolutely required for epiblast formation, while STAT3 is dispensable.
Several studies showed that Nanog and OCT3/4 factors overlap substantially in their target genes, causing sometimes, also by cooperation, the activation or suppression of the expression of target genes. Mapping of the binding sites of OCT3/4 and Nanog in the mouse ESC genome showed that a substantial proportion of the genes were occupied by both factors, in some cases Nanog-OCT3/4 were found to co-localize, in other cases they bound independently (Loh et al., 2006). In the same study it was shown that the OCT3/4-regulated target genes predominantly repress trophectoderm markers whereas Nanog activates the transcription of *Pou5f1* and *Sox2* evidencing a possible role of Nanog in controlling the levels of both OCT3/4 and SOX2 (Fig. 5). These factors in turn control the downstream genes involved in the maintenance of pluripotency or inhibition of differentiation (Loh et al., 2006). Interestingly promoter-sequence analyses showed the presence of a Sox-Oct element on the *Nanog* promoter. Two different groups demonstrated that both OCT3/4 and SOX2 bind to the *Nanog* promoter therefore driving its transcription (Kuroda et al., 2005; Rodda et al., 2005).
ESCs under conventional culture conditions (namely with serum and LIF) are in a dynamic state, that fluctuates between a stable state, in which Nanog expression is high, and an unstable state, where Nanog expression levels are low (Kalmar et al., 2009; Singh et al., 2007). As a consequence ESCs form a heterogeneous population where pluripotent ESCs exhibit a highly variegated gene expression pattern. When induced to differentiate only ESCs with low levels of Nanog expression are able to commit in a stable manner (Chambers et al., 2003). The levels of Nanog expression in an ESC are related to its probability to differentiate or not. In this model the advantage is that in a heterogeneous population, there is always a subpopulation of cells pre-primed for differentiation. Such priming would be an advantage in situations where the cells must be ready within a short period to respond to diverse signaling (Kalmar et al., 2009).
Although there are other transcription factors associated with ESC self-renewal and pluripotency, a large number of studies support the notion that the trio SOX2, OCT3/4 and Nanog are the main regulators that generate and maintain the pluripotent state *in vivo* and *in vitro*. This idea is also corroborated by the fact that all three factors play an essential role in reprogramming somatic cells (Meissner et al., 2007; Okita et al., 2007; Takahashi and Yamanaka, 2006).

3.2 Signaling through cytokine receptors: LIF/gp130 pathways

LIF belongs to the family of interleukin (IL)-6-type cytokines and exerts its effects by binding to a two-part receptor complex, which consists of the low-affinity LIF receptor (LIFRβ) and the glycoprotein 130 (gp130). LIF induces heterodimerization of the LIFRβ and gp130 resulting predominantly in the activation of the JAK/STAT3 (Janus kinase/Signal transducer and activator of transcription signal) transduction pathway, which promotes self-renewal in ESCs. Several studies showed that LIFRβ receptor is not sufficient to mediate the signal to maintain ESCs self-renewal, whereas gp130 is (Niwa et al., 1998; Starr et al., 1997). These results indicate that gp130 is the main component of the activated LIFRβ/gp130 receptor. Interestingly, activation of LIFRβ/gp130 receptor through the binding of LIF leads also to the activation of the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3 phosphate kinase (PI3K) pathways, which together with the JAK/STAT3 pathway are essential for regulating biological responses in ESCs.
In the embryo the epiblast is the transient population of cells from which the fetus is derived. Because of their characteristics, ESCs seem to be the *in vitro* counter part of the epiblast cells *in vivo*. However, in contrast to the LIF dependency of ESCs, early epiblast cells do not require LIF stimulation, since *Lif* null embryos develop normally into later stages (Stewart et al., 1992) and embryos carrying mutations on the LIFRβ and gp130 receptor develop normally, at least until mid-gestation (Li et al., 1995; Nakashima et al., 1999; Ware et al., 1995). Nevertheless, it has been shown that the embryos do express LIF, LIFRβ and the gp130 mRNA indicating a possible function of this pathway also *in vivo*. It has been shown that diapause embryos carrying mutation on the LIFRβ and the gp130 receptors fail to restore normal embryogenesis (Nichols et al., 2001). These findings highlight the absolute requirement for LIF/gp130 signaling in the epiblast during diapause and give also an explanation why

ESCs are LIF dependent. However this is a facultative situation, because the pathway is dispensable for early development without diapause.

3.2.1 JAK/STAT3 signaling and self-renewal

Binding of the cytokine LIF to the receptor results in conformational changes in the intracellular part of the receptor. Cytosolic tyrosine kinases of the JAK family are then recruited to the receptor. The activated receptor phosphorylates the tyrosine residues in the kinase molecule that become docking sites for the STAT3 transcription factors. When bound to the receptor, STAT3 molecules are phosphorylated on the tyrosine 705 (Tyr705) residues and dimerize with another phosphorylated STAT3. The dimers are then translocated to the nucleus in a regulated manner where they bind to promoter and enhancer regions of their target genes (Fig. 6). Between the STAT3 target genes there are the *Socs* genes (Suppressor of cytokine signaling), whose encoded proteins generally act in a negative feedback loop to suppress further STAT3 signaling (O'Sullivan et al., 2007) (Fig. 6).
Although overexpression of STAT3 promotes stem cell self-renewal and maintenance of pluripotency in the absence of LIF and in presence of serum (Cinelli et al., 2008; Matsuda et al., 1999), inactivation of STAT3 in LIF-maintained ESCs promotes spontaneous differentiation (Niwa et al., 1998). Even though these lines of evidence establish STAT3 as an essential component of the LIF-dependent self-renewal in ESCs, the downstream target genes of activated STAT3 have remained elusive. In order to isolate these genes, several studies based on ChIP analysis or on microarray technology have been performed.
Recently Cartwright et al. indicated a role for the transcription factor c-MYC in self-renewal by functioning as a key target of LIF/STAT3 signaling (Cartwright et al., 2005). Like other genes involved in the maintenance of pluripotency, such as *Nanog* (Chambers et al., 2003), *Klf2* (Hall et al., 2009), *Pem/Rhox5* (Cinelli et al., 2008; Fan et al., 1999) and *Pramel7* (Cinelli et al., 2008), constitutive expression of c-MYC renders self-renewal independent of LIF (Cartwright et al., 2005). On the other hand expression of a dominant negative form of c- MYC promotes differentiation (Cartwright et al., 2005). c-MYC is a transcription factor that controls many different biological processes, such as cell proliferation, growth, differentiation and apoptosis. Several studies have shown the importance of this gene in embryonic development, since homozygous deletion of *c-Myc* in the mouse results in embryonic lethality before E10.5 of gestation (Davis et al., 1993).
Not surprisingly, c-MYC is one of the four transcription factors found to be able, together with OCT3/4, KLF4, and SOX2, to reprogram somatic cells into undifferentiated, induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006).
The fact that overexpression of the transcription factor Nanog does not increase significantly the level of phosphorylated STAT3, and *vice versa* the overexpression of STAT3 seems not to affect Nanog expression, leads to the conclusions that *Nanog* is not a direct transcriptional target of STAT3, nor does it regulate STAT3 activity (Chambers et al., 2003). Moreover increased STAT3 activity maintains pluripotency even when Nanog expression is reduced (Bourillot et al., 2009), confirming that these two transcription factors are regulated through different signals. Nevertheless, in a recent study it was shown that 55% of the putative STAT3 target genes display binding sites for Nanog, and 41% of the putative Nanog target genes display binding sites for STAT3 (Chen and Daley, 2008). These results suggest that both transcription factors co-regulate the expression of a large number of target genes, whose expression is involved in the maintenance of the undifferentiated state in ESCs.
Recently Hall et al. showed that OCT3/4 in addition to the LIF/STAT3 signaling activates the Krüppel-factors KLF4 and KLF2 and that their overexpression reduces LIF dependence (Hall et al., 2009). However only KLF2 was able to sustain pluripotency in absence of either LIF or *Stat3*, and was shown to be OCT3/4 induced. KLF4 was shown to be selectively induced by LIF/STAT3 but was not sufficient in absence of the LIF/STAT3 signaling to sustain prolonged ESCs self-renewal. Interestingly, like c-MYC, also KLF4 is one of the four transcription factors that are able to reprogram somatic cells into undifferentiated, self- renewing cells (Takahashi and Yamanaka, 2006).

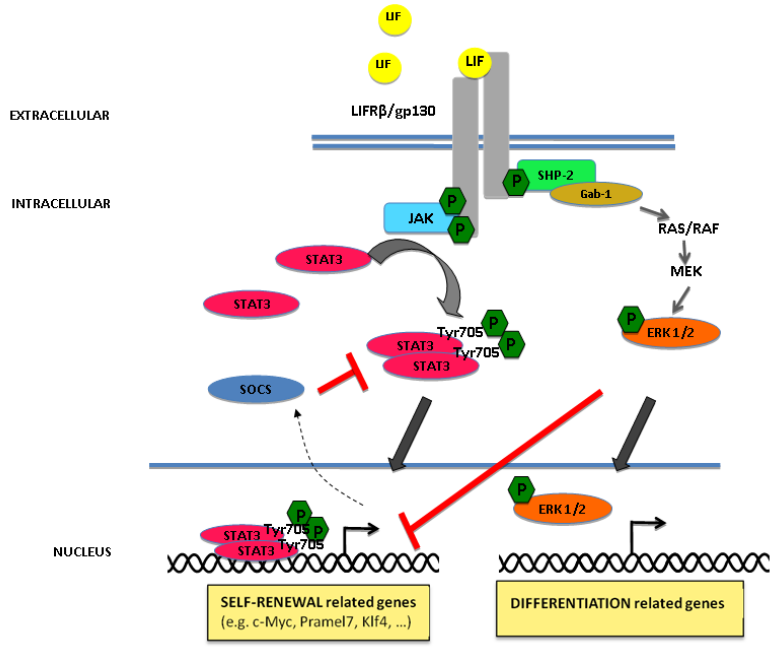


Fig. 6: gp130/LIF dependent STAT3 pathway (see 1.3.2.1) and MAPK/ERK pathway (see 1.3.2.2). Differentiation and self-renewal signaling converge downstream to the LIF receptor. (From Casanova et al., 2011a)

In the molecular mechanisms involved in the maintenance of pluripotency in ESCs extrinsic stimuli converge with intrinsic circuitries in a synergistic manner propagating the undifferentiated and self-renewing state in ESCs. STAT3 is an important regulator of mouse ESC self-renewal and it is known to inhibit differentiation into both mesoderm and endoderm lineages (Ying et al., 2003) by preventing the activation of lineage-specific differentiation programs. However its mechanisms of action remain to be better elucidated.

3.2.2 gp130-dependent activation of the MAPK/ERK pathway

Self-renewal and differentiation converge downstream from the LIFRβ/gp130 receptor. The binding of the cytokine LIF to the heterodimeric receptor activates not only the JAK/STAT3 pathway but also the mitogen-activated protein kinase (MAPK) pathway that culminates in the activation of the extracellular signal-regulated kinases (ERK1/2). Since it has been shown that ERK regulates early differentiation *in vivo* and *in vitro* (Kunath et al., 2007; Nichols et al., 2009b), the balance between self-renewal and differentiation has to be maintained in order to preserve the undifferentiated state of ESCs.
Active gp130 receptor can also associate with the protein tyrosine phosphatase SHP-2 (Fukada et al., 1996), which acts as a positive effector of the MAPK signaling cascade. Interaction between active gp130 receptor and SHP-2 phosphatase induces the recruitment of GAB1. The complexes formed by the gp130 receptor, SHP-2 and GAB1 proteins, through the activation of further kinases (RAS/RAF and MEK) result in the activation of the ERK1 and ERK2 kinases (Takahashi-Tezuka et al., 1998) (Fig. 6). Burdon et al. confirmed that after stimulation with LIF, ERK1 and ERK2 were activated through phosphorylation of SHP-2 (Burdon et al., 1999). Surprisingly, they also showed that suppression of the SHP-2/ERK signaling was not affecting propagation of stem cells, but on the contrary it was enhancing ESCs self-renewal (Burdon et al., 1999). This study indicates that SHP-2/ERK signaling activation is a necessary component of the normal differentiation processes. Differentiation of ESCs into embryoid bodies *in vitro* was associated with an induction of expression of G1 cyclins, a lengthening of the G1 phase and a decrease in the rate of cell division (Savatier et al., 1996). ESCs typically exhibit a short G1 phase of the cell cycle (Rohwedel et al., 1996) and high rate of cell divisions. Entrance into the G1 phase of the cell cycle is a prerogative for cell differentiation.

ERK signaling is known to regulate proliferation and survival of somatic cells (Lloyd, 1998), and *in vivo* phosphorylated ERK has been detected from the 2-cell stage till the blastocyst stage (Wang et al., 2004). Incubation of 2-cell stage embryos with an ERK inhibitor results in a developmental arrest at the four-cell stage embryo; however, normal embryo development can be restored once the inhibitor is removed (Maekawa et al., 2007). In contrast to many mammalian cells, where ERK activity is essential for the cell cycle progression from G0/G1 to S phase (Lewis et al., 1998), during the development from the 2-cell to the 8-cell stage embryo, ERK signaling seems to be essential in the G2/M transition (Maekawa et al., 2007). This confirms that the ERK1/2 pathway is required for progression of early cell division cycles in the preimplantation embryo.
Interestingly, the ERK pathway may be able to inhibit the JAK/STAT3 pathway at the level of STAT3. It has been shown that ESCs knockout for *Shp2* phosphatase after LIF stimulation showed an increased phosphorylation of STAT3 when compared to the wild type cells (Chan et al., 2003). This data supports the evidence that the two pathways seem to converge and thereby determine the choice between self-renewal and differentiation (Fig. 6).
Recently, Ying et al. cultivated ESCs in a serum-free medium containing B27- and N2- supplement in presence of selective small-chemical inhibitors of the FGF receptor and the ERK kinase in combination with a GSK3 inhibitor, the so called 3i or 2i conditions (Ying et al., 2008). While the first two inhibitors are involved in selectively blocking differentiation signals induced by the ERK pathway, the third inhibitor is used for blocking the negative regulation on biosynthetic pathways driven by the GSK3 protein (Ying et al., 2008). This work indicates that by inhibiting differentiation-inducing signals from the MAPK pathway by 3i/2i it is possible to maintain self-renewal in absence of LIF/STAT3 stimulation. This was confirmed by the fact that under these conditions neither STAT3 nor SOCS3 activation was detected. Moreover, it was possible to establish *Stat3* null cells, which did not show morphologically differences when compared with the wild type cells. All the different cell lines established under the 3i conditions expressed the typical pluripotency markers like OCT3/4 and Nanog and were able to contribute to chimera formation and germ line transmission (Ying et al., 2008).

3.2.3 gp130-dependent activation of the PI3K pathway

There are three distinct classes of phosphatidylinositol-3 phosphate kinase (PI3K); members of the class IA family of PI3Ks are activated via the LIF/gp130 receptor (Fig. 7). The products of PI3K transmit the signals through downstream effectors including the serine/threonine protein kinase B (PKB, also known as AKT). AKT has been implicated in many cellular processes like regulation of the cell cycle progression, cell death, adhesion, migration, metabolism and tumorigenesis.
Initially it was shown that this pathway was implicated in the control of proliferation in ESCs (Jirmanova et al., 2002; Takahashi et al., 2003). Paling et al. demonstrated that LIF induced PI3K signal activation in ESCs is involved not only in the regulation of cell proliferation but also in their self-renewal (Paling et al., 2004). ESCs incubated with LIF and a small chemical PI3K inhibitor (LY294002) showed less alkaline phosphatase activity compared to the untreated control cells, indicating a reduced ability of LIF to promote self-renewal. However these cells did not show altered levels of phosphorylated STAT3 when compared to the control cells. This result led to the conclusion that *Stat3* is not a target of PI3K action. The loss of self-renewal and the consequent differentiation of the cells after inhibition of PI3K were explained by an increase in ERK phosphorylation upon LIF stimulation (Paling et al., 2004). These findings are consistent with other studies were it was reported PI3K playing a role in negatively regulating ERK activity in ESCs (Hallmann et al., 2003). Paling et al. demonstrated that self-renewal was restored after incubation with both ERK and PI3K inhibitor, therefore confirming that the regulation of ERK activity by PI3K signaling contributes to the determination of ESCs self-renewal.
Long term treatment of ESCs with the PI3K inhibitor LY294002 was shown to inhibit the proliferation and to induce cell cycle arrest at the G1 phase (Jirmanova et al., 2002). It is known that PI3K/AKT controls cell-cycle regulation: AKT promotes the G1 to S phase transition by facilitating the formation of cyclin/CDK complexes (Brazil et al., 2004). ESCs lack cell-cycle inhibitory mechanisms, which are typical of differentiated cells. Only after differentiation ESCs acquire these mechanisms (Burdon et al., 2002). It is therefore possible that AKT-mediated maintenance of self-renewal in ESCs is due to its

ability to block the cell-cycle inhibitory mechanisms and consequently block differentiation (Watanabe et al., 2006).

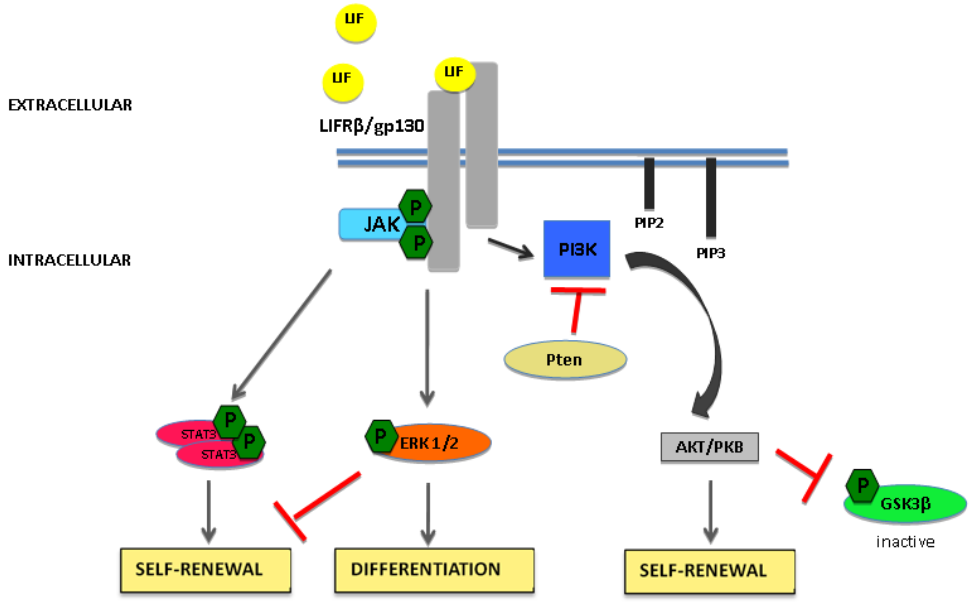


Fig. 7: Summary of gp130/LIF-induced activation of PI3K, STAT3 and ERK1/2 pathways. (From Casanova et al., 2011a)

3.3 Other pathways involved in the maintenance of pluripotency in ESCs

3.3.1 Signaling through the TGF-β pathway: BMP and Nodal/Activin

Transforming Growth Factor β (TGF-β) signaling controls diverse sets of cellular processes, including cell proliferation, recognition, differentiation, apoptosis, and specification of developmental fate, during embryogenesis as well as in mature tissues. Moreover, it has been implicated in the progression of many cancers, functioning both as an antiproliferative and as a tumor-promoting factor. The TGF-β family members bone morphogenetic proteins (BMPs), Nodal and Activin have been implicated in the development and maintenance of various stem cells, including ESCs.
BMP, Nodal and Activin act through the transmembrane type I and type II serine/threonine kinase receptors, leading to their dimerization. The activated receptor recruits SMAD molecules, which carry then the signal from the cell surface to the nucleus.
In serum-free culture, LIF is insufficient to maintain the undifferentiated state of murine ESCs (Ying et al., 2003). On the other hand the overexpression of STAT3 alone is enough to sustain pluripotency in ESCs in a LIF-independent manner, however in presence of serum and feeders (Cinelli et al., 2008; Matsuda et al., 1999). These observations suggest that there must be other factors in the serum or produced by the feeders, which suppress differentiation and concomitantly, efficiently sustain self-renewal in ESCs. One of these signals was shown to be the bone morphogenetic proteins (BMPs). BMPs bind to the Activin receptor-like kinases (ALKs) ALK2, ALK3, and ALK6, and activate the Inhibitor of differentiation (*Id*) genes through the activation of the receptor-regulated SMAD2, SMAD5 and SMAD8 (Ying et al., 2003). In a serum-free culture, ESCs differentiate into neural precursors under the influence of autocrine FGF signal even in presence of LIF (Ying and Smith, 2003) indicating that other signals are required for suppressing neural differentiation. The combination of LIF and BMP4 or BMP2 was found to be sufficient to maintain the undifferentiated state of ESCs in serum-free medium (Ying et al., 2003). However, withdrawal of LIF and retention of BMP causes

differentiation into epithelial-like cells. This lead to the conclusion that the self-renewal response to BMP is dependent on continuous LIF signaling and that the BMP main function is therefore to antagonize the neural differentiation induced by LIF in absence of serum (Ying and Smith, 2003). This was corroborated by the fact that ID overexpression in ESCs enables serum-free self-renewal in the sole presence of LIF. Upon LIF withdrawal, ID overexpressing cells differentiated into non-neuronal precursors, therefore demonstrating that these genes contribute to the ESCs self-renewal by complementing the blockade of other lineages induced by STAT3.
These findings clearly indicate that the cooperation between LIF/STAT3 and BMP/SMAD pathway is required for maintaining ESCs in serum-free media. Nevertheless the balance has to be critically regulated, since depletion of the LIF/STAT3 signaling induces BMP-dependent promotion of differentiation and overexpression of SMAD1/4 overrides the effect of LIF and causes non-neural differentiation (Ying and Smith, 2003). Recapitulating, high expression of BMP leads to differentiation into mesoderm and endoderm lineages, whereas neural differentiation is suppressed. On the other side, low level of BMP also induces differentiation into mesoderm. Therefore, support of pluripotency by BMP pathway is highly dose-dependent and needs to be counter-regulated by STAT3 signaling.

Nodal/Activin signaling has been shown to be essential *in vivo* for the induction of mesoderm and endoderm lineages and for the determination of the left-right axis during embryogenesis. Nodal knockout show a reduced epiblast cell population that display very low expression of the pluripotency marker OCT3/4 and arrest the development before gastrulation (Robertson et al., 2003).
Nodal expression in mouse ESCs is high, and was found to build an active signaling together with SMAD2. Thus, stimulation of ESCs with Activin or Nodal leads to an increase in SMAD2 phosphorylation and a higher ESCs proliferation, whereas inhibition of SMAD2 activation reduces cell proliferation (Ogawa et al., 2007).
In serum-containing medium, both BMP and Nodal/Activin pathways are autonomously activated in ESCs. Ogawa and colleagues showed that after overexpression of the inhibitors SMAD6 and SMAD7, ESCs proliferation significantly decreased, being the effect in SMAD7 transfected ESCs much more dramatic. The SMAD7-dependent inhibition of cell proliferation was reversible after excision of the transgene; moreover this was not affecting ESCs pluripotency since injection of the SMAD7-reverted cells produced live chimeras (Ogawa et al., 2007). In serum-free medium the SMAD7 induced blockade of proliferation is more reduced, leading to the conclusion that soluble TGF-β-related molecules in the serum are also involved in the SMAD7-dependent growth inhibition (Ogawa et al., 2007). Further analyses demonstrated that ESCs autonomously activate Nodal/Activin signaling by producing these ligands in serum-free conditions; in presence of serum the soluble TGF-β-related molecules might increase the endogenous Nodal/Activin activity leading to an enhanced cell proliferation (Ogawa et al., 2007).
If the involvement of the Nodal/Activin pathway in maintaining mouse ESCs has still not been completely elucidated, this is not the case for human ESCs (hESCs), where it plays a fundamental role in the maintenance of pluripotency. Like mouse ESCs, hESCs do express all the components of the LIF/STAT3 pathway but in contrast to the mouse cells, they cannot be maintained pluripotent in presence of LIF (Humphrey et al., 2004). This indicates that signaling through this pathway is insufficient to prevent differentiation of hESCs and indicates the existence of other pathways involved in the regulation of pluripotency in hESCs. Interestingly, mouse epiblast stem cells derived from the E5.5-E6.5 postimplantation embryos can also not be maintained undifferentiated in presence of LIF or BMP4. Like hESCs, mouse epiblast stem cells require FGF4 and Nodal/Activin signaling for self-renewal (Brons et al., 2007; Tesar et al., 2007). In conclusion, even though all the components of the Nodal/Activin pathway are highly expressed in both hESCs and mouse ESCs, the outcome of these signaling is different in the two species.

3.3.2 The canonical Wnt pathway

The WNT pathway plays crucial roles in controlling genetic programs of embryonic development and adult homeostasis. WNT signals are transduced depending on their functions through different receptors and members: The canonical WNT pathway is known to be involved in transmitting signals

for cell fate determination, whereas the non-canonical WNT pathway is involved in controlling cell movements and tissue polarity.
Canonical WNT signaling starts when the extracellular WNT ligand binds members of the Frizzled and LDL receptor family. The main player of the cascade is the cytoplasmic protein β-catenin. When the WNT ligand activates the pathway, β-catenin translocates to the nucleus where it interacts with other members of the signaling pathway for activating target genes.
The tumor suppressor adenomatous polyposis coli (APC), AXIN and the glycogen synthase kinase 3β (GSK3β) are the components of the cytosolic destruction complex responsible for the proteasomal β-catenin degradation in absence of WNT ligand.
Several publications support the role of the WNT/β-catenin pathway in maintaining pluripotency in ESCs, although the precise mode of action needs still to be clarified. It has been shown that the feeders used for the cultivation of ESCs represent a potential source of WNT ligands, or that ESCs conditionally expressing a constitutive active form of β-catenin were able to maintain the undifferentiated state in a LIF-independent manner (Hao et al., 2006). This study demonstrated that the WNT-mediated maintenance of pluripotency was depending on β-catenin stabilization. Moreover, constitutive activation β-catenin induced an upregulation of STAT3 mRNA and protein. In serum-free medium the WNT pathway is not sufficient for sustaining ESCs self-renewal, however if 10 U/ml of LIF are added to the medium, ESCs form compact colonies and show OCT3/4 expression suggesting a synergistic effect of both pathways; the WNT pathway effectively upregulating STAT3 mRNA and the LIF pathway phosphorylating the protein and finally activating STAT3 target gene expression (Hao et al., 2006).
Nevertheless, in *β-catenin* knockout ESCs the expression of the core pluripotency factors Nanog, OCT3/4 and SOX2 is still present indicating that β-catenin regulates the expression of several stemness genes, but is not directly required for maintenance of pluripotency (Anton et al., 2007).
Recently it has been shown that β-catenin can form a complex with and modulate the activity of OCT3/4 (Kelly et al., 2011). *Gsk3α/β* knockout ESCs expressing a dominant-negative form of TCF1 or TCF4 had attenuated β-catenin/TCF gene transactivation, but surprisingly were not rescued with respect to their ability to differentiate into neurectoderm and self-renewed in the absence of exogenous factors (Kelly et al., 2011). It was therefore postulated that the contribution of β-catenin in the maintenance of pluripotency in ESCs is exerted through a TCF-independent mechanism (Kelly et al., 2011).
Despite the recent findings the role of the WNT pathway in regulating pluripotency in ESCs is still not clear and further analysis would be needed in order to clarify its real contribution in the pluripotency-related processes.

4. Aim of the work

The overall goal of this thesis was to gain knowledge on the molecular mechanisms that control stemness in the embryonic stem cells. The work was divided into two different projects: the first was focused on the function of pluripotency related genes in ESCs and the second project consisted in a genome wide expression analysis of mouse and rat preimplantation stage embryos.

> *Project number 1: Functional characterization of newly identified pluripotency-related genes in murine ESCs*
>
> *Project number 2: Cross-species genome wide expression analysis of late mouse and rat preimplantation embryo development*

4.1 *Project 1*: Functional characterization of newly identified pluripotency-related genes in murine ESCs

4.1.1 Expression profiling in transgenic FVB/N embryonic stem cells overexpressing STAT3

The aim of this part of the study was to analyze the function of selected genes, which were differentially regulated in the STAT3 overexpressing cells (Cinelli et al., 2008). To achieve this goal, vectors containing the ORF of the genes *Hexokinase II*, *Pem/Rhox5*, *Dppa3/Stella*, *Pramel7*, and *Pramel6* have been cloned for their functional characterization in ESCs. ESCs overexpressing these genes have been generated and analyzed for their ability to be propagated in the absence of LIF in the medium.

Based on these preliminary results we selected the genes *Pramel7* and *Pramel6* for further characterizations.

4.1.2 Pramel7 mediates LIF/STAT3 dependent self-renewal in embryonic stem cells

Given that the candidate genes showed a differential expression in transgenic FVB/N ESCs, we hypothesized they could play a role in the stabilization of pluripotency in ESCs (Cinelli et al., 2008). Therefore, the aim of this part of the project was to extensively analyze and characterize the function of *Pramel6* and especially of *Pramel7* in the maintenance of pluripotency in murine ESCs.

For this purpose transgenic ESCs were generated, which overexpressed in a constitutive manner either Pramel6 or Pramel7. The capability of both genes to maintain ESCs in an undifferentiated state was analyzed by cultivating these cells in the absence of LIF on *Lif* knockout feeders. Moreover, the pluripotent state of the transgenic cells, maintained through the sole overexpression of Pramel6 and Pramel7 was investigated by generating chimeric mice. The confirmation of their undifferentiated and pluripotent state *in vitro* was investigated by immunoassay and real-time PCR analysis.

The differentiation potential of Pramel7 and Pramel6 overexpressing cells was monitored *in vitro* (embryoid bodies formation, neural differentiation) as well as *in vivo* (teratoma formation, blastocyst injections).

Finally the molecular mechanisms which drive the transcription of *Pramel7* were investigated in transgenic and wild type ESC lines.

4.2 *Project 2*: Cross-species genome wide expression analysis of late mouse and rat preimplantation embryo development

The aim of this project was to identify differentially regulated mechanisms in the mouse and the rat morula and blastocyst stage embryos with the purpose of increasing the knowledge about the regulation of pluripotency in rat ESCs.

With this intention, morulae, blastocysts, and ICMs were collected from both species and a whole genome microarray analysis was performed.
In a first part of the study we analyzed separately for the mouse and for the rat, the differentially expressed genes in the three cell populations. We next performed a cross-species analysis in order to highlight similarities and differences between the gene expressions in the two species. Important signaling pathways were investigated and compared between the mouse and the rat, as well as the expression patterns of gene families.
Finally the expression of genes known to be involved in the maintenance of ESCs *in vitro*, were analyzed and compared in the two species.

B. RESULTS

1. Functional characterization of new pluripotency-related genes in murine ESCs

Despite 30 years of research and the discovery of many factors indispensable for maintaining and generating pluripotent ESCs, still many questions are open. The LIF/STAT3 pathway plays a crucial role in the maintenance of pluripotency in ESCs. Although lines of evidence establish STAT3 as an essential component of the LIF-mediated self-renewal in ESCs, the downstream target genes of activated STAT3 have remained elusive. Moreover, as mentioned before, the genetic background strongly impairs the efficiency of establishing genuine ESCs. So that for many years, ESCs of proven ability to colonize the germ-line have been obtained at very low frequency in only a few mouse strains other than 129 (Simpson et al., 1997).

In the next chapters are reported two studies, where we identified new pluripotency-related genes upon overexpression of STAT3 in murine ESCs.

The resulting papers are attached as ***Annex*** at the end of this Thesis.

1.1 Expression profiling in transgenic FVB/N embryonic stem cells overexpressing STAT3

Besides finding the right culture conditions, manipulating the expression of specific genes known to safeguard the pluripotent state of ESCs, represents a good method for generating stable ESC lines.

Matsuda et al. demonstrated that overexpression of the transcription factor STAT3 maintained the undifferentiated state of 129/SvJ-derived ESCs (Matsuda et al., 1999).
The authors generated a chimeric STAT3-estrogen receptor (STAT3-ER) composed of the entire coding region of STAT3 and the ligand-binding domain of the estrogen receptor. Dimerization of the chimeric STAT3 was activated after treatment with the estrogen derived 4-hydroxy-tamoxifen (4-OHT). ESCs cultivated in presence of 4-OHT were able to self-renew in the absence of LIF (Matsuda et al., 1999).

We adopted the same system used in the study from Matsuda et al. and we were able to generate germline competent ESCs from the non-permissive FVB/N mouse strain by overexpressing STAT3. This indicates that overexpression of STAT3 is not only sufficient to maintain already established ESCs pluripotent, but is also able to increase the efficiency of derivation of ESCs from the ICM of strains where normally derivation of viable ESCs is otherwise impaired. Moreover, we compared by microarray analyses the gene expression differences between FVB/N wild type ESCs cultivated in presence of LIF and STAT3 overexpressing transgenic ESCs cultivated in presence of tamoxifen and identified potential new STAT3 target genes.

This study was published in 2008. The paper (Cinelli, P.; Casanova, E. A.; Uhlig, S.; Lochmatter, P.; Matsuda, T.; Yokota, T.; Rülicke, T.; Ledermann, B. & Bürki, K. (2008). Expression profiling in transgenic FVB/N embryonic stem cells overexpressing STAT3. BMC Dev Biol, 8, 57) is reprinted and attached at the end of this Thesis (***Annex 1***).

1.2 Pramel7 mediates LIF/STAT3 dependent self-renewal in embryonic stem cells

The central role of the LIF/STAT3 pathway in maintaining pluripotency in ESCs cultures has been repeatedly demonstrated in the last years (Cinelli et al., 2008; Hall et al., 2009; Matsuda et al., 1999; Schoonjans et al., 2003; Yang et al., 2010). Nevertheless, only few STAT3 target genes have been identified.

In our previous work we identified a set of genes with differential expression upon STAT3 overexpression. The generated transgenic cells possessed characteristics typical of pluripotent ESCs and showed germline transmission. These properties were not present in the wild type ESCs. We therefore reasoned that some of the genes differentially expressed in the transgenic cells might play a role in controlling pluripotency.

We concentrate our efforts on the *Pramel7* gene, which was found to be upregulated in the FVB/N transgenic ESCs (Cinelli et al., 2008). In parallel we analyzed another member of the Pramel family, the *Pramel6* gene, which was also upregulated in the previous microarray study.
We first addressed the question whether *Pramel7* and *Pramel6* are involved in maintaining ESCs pluripotent. For this purpose we generated clones overexpressing one or the other gene and cultivated these cells in the absence of LIF. The sole overexpression of Pramel7 upon withdrawal of LIF was able to sustain self-renewal in ESCs. On the contrary, Pramel6 seemed not to play a central role in maintaining ESCs undifferentiated. We therefore further extensively investigated the function of *Pramel7* only.
Knock down experiment for Pramel7 induced clear differentiation of wild type ESCs, despite the presence of LIF in the medium. Moreover, also in STAT3 overexpressing cells the silencing of Pramel7 induced differentiation of the ESCs, confirming the importance of this gene in maintaining ESCs in a self-renewing and undifferentiated state. Of importance, Pramel7 overexpression completely abolished differentiation of ESCs.
Furthermore, we demonstrated that *Pramel7* transcription is directly controlled by STAT3 transcription factor. Interestingly, we found that the LIF/PI3K pathway, through the activation of the GSK3β, is involved as well in regulating *Pramel7* transcription. This finding showed for the first time a common target of both LIF/STAT3 and LIF/PI3K pathways.

These results have been published in *Stem Cells* Journal. The paper (Casanova E. A.; Shakhova O.; Patel S. S.; Asner I. N.; Pelczar P.; Weber F. A.; Graf U.; Sommer L.; Bürki K. and Cinelli P. (2011). Pramel7 mediates LIF/STAT3 dependent self-renewal in embryonic stem cells. *Stem cells* 29(3):474-85.) is reprinted and attached at the end of this Thesis (***Annex 2***).

2. Cross-species genome wide expression analysis of late mouse and rat preimplantation embryos

The molecular events that take place during mammalian preimplantation development represent a good model for studying regulatory networks that determine cell fate decisions. Of particular interest is the switch between morula and blastocyst stages, the first differentiation event of embryogenesis, a period where pluripotent cells are formed. Understanding how this population of cells is generated and maintained is of fundamental importance also for understanding the molecular mechanisms that control pluripotency in ESCs.

As mentioned in the Introduction part of this Thesis, the rat represents in many research fields an optimal animal model for studying human diseases. Nevertheless, the lack of pluripotent rat ESCs for many years bound the researchers to work with mouse models, where indeed ESCs were available. Only recently, almost 30 years after the establishment of the first murine ESC line, genuine rat ESCs have been generated (Buehr et al., 2008; Li et al., 2008). Nevertheless, these cells have to be cultivated under different conditions compared to the murine ESCs, and seem to be more sensitive to differentiation stimuli. Thus, murine ESCs differ from the rat ESCs in their molecular mechanisms.

We reasoned that a comparison of the whole genome expression in the late preimplantation embryo stages could be of advantage for elucidating the molecular mechanisms involved in the generation and maintenance of the pluripotent cells. Moreover, we aimed at the identification of similarities and differences in the gene regulation between the mouse and the rat preimplantation embryos, which could explain the differences observed in vitro for the ESCs between the two species. Therefore, we isolated morula, blastocyst, and ICMs from both the species and we performed microarray analysis.

We first performed a general investigation of the gene expression in the two species separately. We then investigated and compared the gene expression in the mouse and in the rat for genes involved in 11 signaling pathways, that we selected from GeneGo. Moreover, we performed a cross species analysis also with 11 families of genes known to be important during development as well as in the maintenance of pluripotency.

With this study we were able to show for the first time that many molecular processes as well as signaling pathways are strongly differentially regulated in the mouse and in the rat morula and blastocyst. The knowledge that important factors are differentially expressed in the mouse and in the rat ICM, for instance, could be of advantage for improving the derivation and maintenance of rat ESCs.

These results have been recently submitted. The manuscript (Casanova Elisa A.; Okoniewski Michal J.; Cinelli Paolo. Cross-species genome wide expression analysis of late mouse and rat preimplantation embryos) is attached at the end of this thesis (***Annex 3***).

C. DISCUSSION AND OUTLOOK

Due to the fundamental characteristics of ESCs, developmental biology, regenerative medicine and cancer biology are more and more interested in understanding the molecular mechanisms controlling stem cells. Even though a lot of efforts have been made in the past years to elucidate the factors that regulate stem cell self-renewal and pluripotency, the precise mechanism of how these processes are regulated remains largely unknown. The identification of new genes responsible or involved in the maintenance of pluripotency will be of advantage not only for a better understanding of how pluripotency is maintained but also for improving the establishment of ESCs from non-permissive strains and species.
Moreover, a major goal of ESC research is to elucidate the decision-making processes in lineage commitment and cell type differentiation of pluripotent cells. Therefore, understanding and controlling cell fate determination remains a major challenge for the efficient *in vitro* analysis of gene function but also for defining which conditions are optimal for prolonged cultivation of robust pluripotent ESCs.
As mentioned before, for many years the empiric approaches based on experience with mouse ESCs have not resulted in the establishment of stable rat ESC lines. Therefore, we have become interested in the analysis of the molecular pathways connected with pluripotency in ESCs in order to isolate factors that could be useful for improving the establishment and the stabilization of ESCs from non-permissive mouse strains and from the rat.

This work was divided into two major projects: The first was an analysis of new identified genes involved in the maintenance of pluripotency in murine ESCs; the second project was a whole genome study of preimplantation embryos from the mouse and the rat. Both projects aimed at dissecting the molecular mechanisms that regulate pluripotency, the first *in vitro* in the ESCs whereas the second *in vivo* in the morula and blastocyst stage embryos.

1. Functional characterization of newly identified pluripotency-related genes in ESCs (Project 1)

The starting point for this project was the demonstration that the activation of the LIF/STAT3 pathway during the cultivation of blastocysts supports ICM outgrowth and clearly favours the establishment of new ESC colonies in the non-permissive FVB/N mouse strain (Cinelli et al., 2008). Interestingly, the expression of the classical pluripotency markers (OCT3/4, SSEA-1, and alkaline phosphatase) was similar in the transgenic FVB/N ESCs and in the wild type FVB/N ESCs. Nevertheless, only the transgenic cells were able to participate to the embryo development and show germ line transmission, indicating that they were authentic pluripotent ESCs (Cinelli et al., 2008). Even though these lines of evidence establish STAT3 as an essential component of the LIF-dependent self-renewal in ESCs, the downstream target genes of activated STAT3 had remained elusive.
Due to the pluripotent characteristic of the transgenic FVB/N ESCs, not observed in the wild type cells, a microarray study was performed in order to identify new potential STAT3 target genes. The differentially expressed genes obtained from this study represent potential critical factors involved in the determination of the pluripotent state.

Thus, the aim of this part of the study was to analyze the function of selected genes, which were differentially regulated in the STAT3 overexpressing cells (Cinelli et al., 2008). The overexpression of

genes that are considered potential partners in maintaining and stabilizing pluripotency in cultured ESCs is a powerful tool for elucidating their function. Therefore, based on preliminary results and because their function was largely unknown, we generated ESCs overexpressing either the gene *Pramel7* or *Pramel6* for elucidating their role in the maintenance of pluripotency in ESCs.

Pramel7 and *Pramel6* are part of the Pramel family that contains a large number of members, with temporal and spatial expression highly restricted to well-defined stages of the preimplantation embryonic development. These genes are similar to the preferentially expressed antigen in melanoma (PRAME) protein and are therefore named as *PRAME-like* (Pramel) genes. 18 *PRAME-like* genes and 15 pseudogenes were predicted in the orthologous region of mouse chromosome 4 (Birtle et al., 2005). Members of the Pramel family are also the oogenesins, which are specifically expressed in the oocyte (Dadé et al., 2003) but they have been detected also in the nuclei of the late one-cell- to early two-cell stage embryos (Minami et al., 2003). The expression of the *Pramel4* gene for instance is restricted to the 8-cell stage and disappears almost completely in the blastocyst (Zeng et al., 2004), whereas the one of *Pramel3* is detected only in the two-cell stage embryos.
The expression of *Pramel6* and *Pramel7* is restricted to the preimplantation embryos (Bortvin et al., 2003). Precisely, *Pramel6* is expressed in all the cells of the morula and blastocyst, whereas the expression of *Pramel7* is restricted to the inner cells of the morula and to the ICM cells of the blastocyst (Cinelli et al., 2008). Thus, a large body of evidence suggests that the members of the Pramel gene family might play *in vivo* a role in orchestrating the switch between the different preimplantation developmental stages.
The expression patterns *in vivo* of *Pramel6* but especially of *Pramel7*, in addition to the fact that both are upregulated in ESCs upon STAT3 overexpression (Cinelli et al., 2008), makes these genes interesting candidates for the analysis of their function in the maintenance of pluripotency in ESCs.

Our results demonstrate that *Pramel7* is a new direct STAT3 target gene, fundamental for the LIF-mediated maintenance of pluripotency and for the inhibition of differentiation (Casanova et al., 2011b). The JAK/STAT3 pathway was shown to be essential and sufficient in mouse ESCs to mediate LIF signals thereby contributing to the maintenance of pluripotency (Matsuda et al., 1999; Smith et al., 1988). Even though a complete bypass of LIF signaling is possible under certain circumstances (Ying et al., 2008), optimal self-renewal is obtained by combination of LIF and 2i. These observations together with our findings confirm the LIF/STAT3-pathway as an essential component in ESCs.
This is of interest also because overexpression of Pramel7 completely abolishes the capability of ESCs to differentiate *in vitro* as well as *in vivo* (Casanova et al., 2011b). Curiously, the overexpression of Nanog in ESCs does not impair the generation of teratomas composed from all the derivatives of the three germ layers (Casanova et al., 2011b), leading to the assumption that these two genes act differently in the processes that regulate pluripotency. Taken together, our data demonstrated that Pramel7 represents a central and essential knot in the signaling network regulating pluripotency and self-renewal in ESCs.

However, although we were able to confirm that *Pramel7* is a direct new target of STAT3 and actually that the LIF/STAT3 mediated maintenance of pluripotency is highly Pramel7 dependent, the exact mechanisms of function of this gene remain elusive.
We have elaborated a hypothetical mechanism (Fig. 8), in which STAT3 together with the kinase GSK3β drive the expression of *Pramel7* (Casanova et al., 2011b). Moreover, the fact that upon forced expression of Pramel7 ESCs fail to differentiate and under some conditions die, lead to the assumption that *Pramel7* transcription has to be strictly regulated *in vitro*. The biological function of Pramel7 and the mechanisms by which this protein is able to maintain pluripotency in ESCs are still unknown. Our data indicate that Pramel7 could maintain pluripotency through direct repression of differentiation by preventing the phosphorylation of the ERK protein (Fig. 8). Nevertheless further analyses will be necessary to confirm these observations.

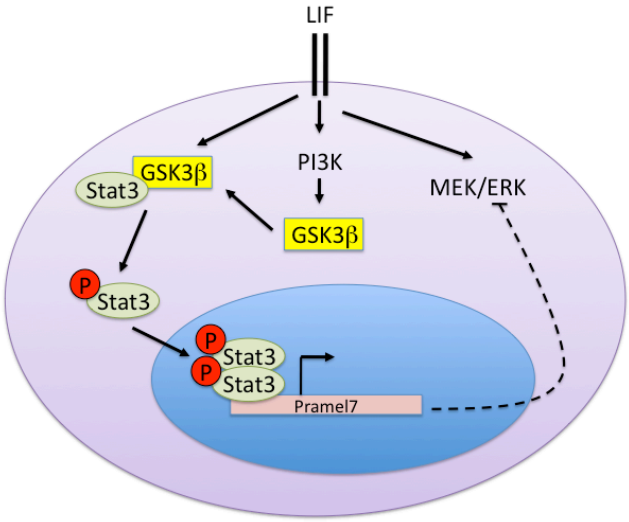


Fig. 8: Hypothetical transcriptional mechanisms, which drive *Pramel7 expression*. Schematic representation of Pramel7 transcription directly controlled by the LIF/STAT3-pathway but also by the LIF/PI3K-pathway. LIF/gp130 receptor leads to the activation of three different pathways: the LIF/STAT3, the LIF/PI3K/GSKβ3 and the LIF/MEK/ERK-pathway. *Pramel7* transcription is directly controlled by the transcription factor STAT3 whereas its phosphorylation is probably regulated by the GSK3β kinase. The combinatorial effect of LIF/STAT3 and GSK3β drives and controls *Pramel7* transcription, which in turn blocks phosphorylation of ERK and therefore ESCs differentiation. (From Casanova et al., 2011b)

Even though in the past years many different key factors could be identified, how they interact with each other and control the pluripotent cell identity is still largely unknown. Search for recognizable domains in the ORF of *Pramel7* revealed the presence of leucine-rich repeats (LRRs). The presence of LRR domains and the fact that aminoacid sequence analysis did not reveal the existence of any conserved domains typical for transcriptional factors indicates that the *Pramel* gene family might not directly regulate gene transcription but rather act via protein-protein interaction. It was therefore decided to identify possible interaction partners of Pramel7 by a yeast two- hybrid screen. Bait containing the full-length sequence of Pramel7 fusioned to LexA was generated and used to screen an ESC library specifically prepared for this analysis. Three independent clones could be isolated, all containing the coding sequence of *Uhrf1*.
The *Uhrf1* gene encodes a member of RING-finger E3 ubiquitin ligase and was shown to be involved in processes like genome methylation (Karagianni et al., 2008) and histone modifications (Citterio et al., 2004). Furthermore, Uhrf1 was shown to directly target different promoters and regulate the protein expression levels of cell cycle regulators like pRB (Jeanblanc et al., 2005).

The result obtained from the yeast two- hybrid screen generated a large body of open questions and possible hypothesis about the function of Pramel7 and its involvement in epigenetic processes. These topics are now further analyzed in the laboratory.

In conclusion, our data show that the combined activity of STAT3 and GSK3β controls *Pramel7* transcription, which in turn regulates the phosphorylation of ERK leading to the inhibition of ESC differentiation. Accordingly, Pramel7 ablation causes ESC differentiation, whereas its overexpression sustains long-term self-renewal in the absence of LIF. These observations prove Pramel7 as an essential factor of the signaling network regulating pluripotency and self-renewal in ESCs (Casanova et al., 2011b).
Nevertheless, further analyses aimed at the characterization the mechanisms involved in the Pramel7-dependend maintenance of pluripotency in ESCs are needed.

The gene expression analysis performed in the STAT3 overexpressing ESCs highlights the differential expression of other interesting genes, which might contribute in the maintenance and stabilization of pluripotency in ESCS. One promising candidate is the gene *Pem/Rhox5*, which was upregulated in ESCs upon STAT3 overexpression (Cinelli et al., 2008). Its expression *in vivo* is restricted in the morula and the whole blastocyst stage embryos and after implantation it is restricted to the extraembryonic tissues. Furthermore, *Pem/Rhox5* was shown to be expressed in ESCs, primordial germ cells and teratocarcinoma cells (Payer et al., 2003).
Similar to Pramel7, the overexpression of Pem/Rhox5 in ESCs is sufficient for maintaining the cells in a self-renewing and undifferentiated state in the absence of LIF in the medium (Cinelli et al., 2008). This is in accordance with a previous study where it was shown that embryoid bodies overexpressing this gene were not able to differentiate (Fan et al., 1999). Interestingly, the same authors declared that teratomas generated from ESCs overexpressing Pem/Rhox5 consisted in only undifferentiated embryonal carcinoma-like cells. In contrast to this study, we obtained out of four injections into NOD/SCID mice of Pem/Rhox5 overexpressing cells, only one teratoma that contained differentiated tissue similar to the teratomas obtained from the wild type ESCs (Casanova et al., 2011b). Thus, Pem/RHox5 overexpressing cells show at least to some extent a similar differentiation defect like the Pramel7 overexpressing cells. Nevertheless, this experiment should be repeated in order to clarify the function of *Pem/Rhox5* in differentiation processes. Two previous studies suggested different potential function of this gene. One hypothesis is that *Pem/Rhox5* first helps to maintain the undifferentiated state of the cells, and in a second step promotes a defined cell population of undifferentiated stem cells for differentiation into extra embryonic lineages (Fan et al., 1999). Another group proposed an alternative mechanism of function in which *Pem/Rhox5* directs early differentiation to specific lineages, but does actively maintain the undifferentiated state (Sasaki et al., 1991).
In sum, the clarification of the function of *Pem/Rhox5* in the maintenance of pluripotency needs further analysis.

The ESCs overexpressing Pem/Rhox5 in a constitutive manner are already available in the laboratory. These cells can be further analyzed in a similar way as for the Pramel7 overexpressing cells. Upon Cre-recombination the overexpression of Pem/Rhox5 can be reduced to the wild type level. This analysis would allow the clarification if ESCs maintained only through the overexpression of Pem/Rhox5, in the absence of LIF, retain their pluripotent potential upon injection into a blastocyst. Furthermore, also available in the laboratory are transgenic mice generated in previous works which express in a tamoxifen-inducible manner Pem/Rhox5. By using the two systems available in the laboratory, the clarification of the function of *Pem/Rhox5* can be analyzed both *in vivo*, during embryogenesis, but also *in vitro*, in ESCs. Furthermore, the analysis could be performed in parallel in mouse as well as in rat ESCs.

2. Cross-species genome wide expression analysis of late mouse and rat preimplantation embryo development (Project 2)

The aim of this project was to identify differentially regulated mechanisms in the mouse and the rat morula and blastocyst stage embryos with the purpose of increasing the knowledge about the regulation of pluripotency in rat ESCs.

In the year 2007, when we started this project, it was not possible to establish authentic rat ESCs. Moreover, all the attempts to generate rat ESCs by using culture conditions established for murine ESCs failed. Only one year later (2008) the first report was published where it was demonstrated the generation of pluripotent rat ESCs by using the 2i culture conditions (Buehr et al., 2008; Li et al., 2008). Even though the 2i conditions are also used for establishing mouse ESCs, some discrepancies between the establishment of rat or mouse ESCs are still present. In fact, rat ESCs are successfully generated with the 2i conditions only when feeders and LIF are present. These prerequisites are not

necessary for establishing murine ESCs, which are routinely cultivated and established in feeders- and LIF-free conditions. Thus, even these findings corroborated the hypothesis that on the molecular level pluripotency is regulated in a different way in the two species. Since *in vivo* pluripotent stem cells transiently exist only in morula and blastocyst stage embryos, we considered these cells as a reliable source for gaining insight into the molecular processes that regulate pluripotency in the mouse and in the rat.

The first part of this project was to isolate morula and blastocyst stage embryos from both species, and with part of the blastocysts to perform immunosurgery in order to separate the ICM from the trophoblast cells. Moreover, the pool of collected embryos had to be large enough to allow the extraction of sufficient amounts of RNA for performing the microarray analysis.
In the case of the isolation of the mouse embryos we could superovulate the females so that with high efficiency we reached the adequate number of embryos in a reasonable period of time. To mention is the fact that embryos were isolated weekly and then stored at -80°C till the final numbers of embryos were collected. The major problem was to preserve the RNA present in the embryos during the whole "collection time" in a good manner, in order to then isolate high quality RNA for the microarray analysis.
As mentioned before, for the mouse females an established protocol was available so that we could collect the embryos in a short time, and after adjusting the storing conditions, we were able to extract an adequate amount of RNA in a good quality.
In contrast, the collection of the rat embryos took longer time compared to the same procedure performed for the mice. Initially, we followed a previously established protocol in the laboratory used for superovulating rat females (i.p. injections of 15IU pregnant mare's serum gonadotropin (PMSG) and 15IU human chorionic gonadotropin at 48h intervals). Nevertheless, no embryo could be isolated observing this protocol. A possible explanation is that this protocol has been established for the collection of cumulus–oocyte complexes, a much more early stage compared to the morula and blastocyst stage we wanted to isolate. Moreover, the effect of the hormones used increase the number of mature oocyte but do not prepare the female for a real pregnancy, this means that the so-obtained embryos cannot implant into the uterus wall. For this reason also for the collection of mouse embryos, we used to isolate the embryos at the morula stage, just before they reached the uterus.

We next followed a new protocol developed by Popova et al. (2005) but also in that case, although we were able to isolate morulae, we did not observe a real increase in the numbers of embryos. Moreover, the quality of the so-obtained embryos was very low. Thus, since we were not able to induce the oestrus of the females by using the superovulation technique, we recurred at the natural ovulation. The impossibility to superovulate the rat females had as a consequence that we sacrificed a larger numbers of rats for collecting all the embryos needed for the study. Moreover, the impossibility to induce the oestrus in the females caused a large delay in the accomplishment of the collection of all the rat embryos.
Rat females were checked every afternoon for the oestrus by measuring the vaginal impedance, which fluctuates markedly across different phases of the cycle (Ramos et al., 2001). The females in oestrus were mated for 12h with the males, and the day after was considered as day E0.5 of pregnancy. Pregnant females were sacrificed either at day E4 or at day E4.5, the former for isolating morula and the latter for isolating blastocyst stage embryos. Due to the bigger size of the rat uteri, a larger volume of medium was used for flushing the embryos. This rendered the identification of the embryos much more difficult than in the case of the mouse.
The collection of all the morula, the blastocysts, and the ICMs from the rat lasted for almost one year. Finally an adequate number of embryos was obtained and the quality of the extracted RNA was good as well, so that we could perform the microarray analysis also for the rat embryos.

From our genome wide cross species analysis in the three cell populations (morulae, blastocysts, and isolated ICMs), we were able to highlight differential expression of important genes like *β-catenin*, *c-Myc*, or *Smad4* (see Casanova et al., submitted Manuscript). All of them are part of essential signaling pathways that play critical functions during the embryo development, and their differential regulation strongly affects the activation or inhibition of the pathways in which they are involved. Some of the genes identified with a differential expression in the two species are known to be important factors in

the maintenance of pluripotency in ESCs, like it is the case for instance for *Sox2* or *Stat3,* and would therefore represent interesting candidate to further analyze *in vitro* in the rat ESCs.
Moreover, our study represents also the first genome wide gene expression analysis in the morula and blastocyst of the rat and shows a new example of statistical approach for cross species analysis that could be applicable also for other species. The so-obtained data allows highlighting the species-specific behaviour of genes within important pathways and families through the creation of own gene networks (Fig. 9).

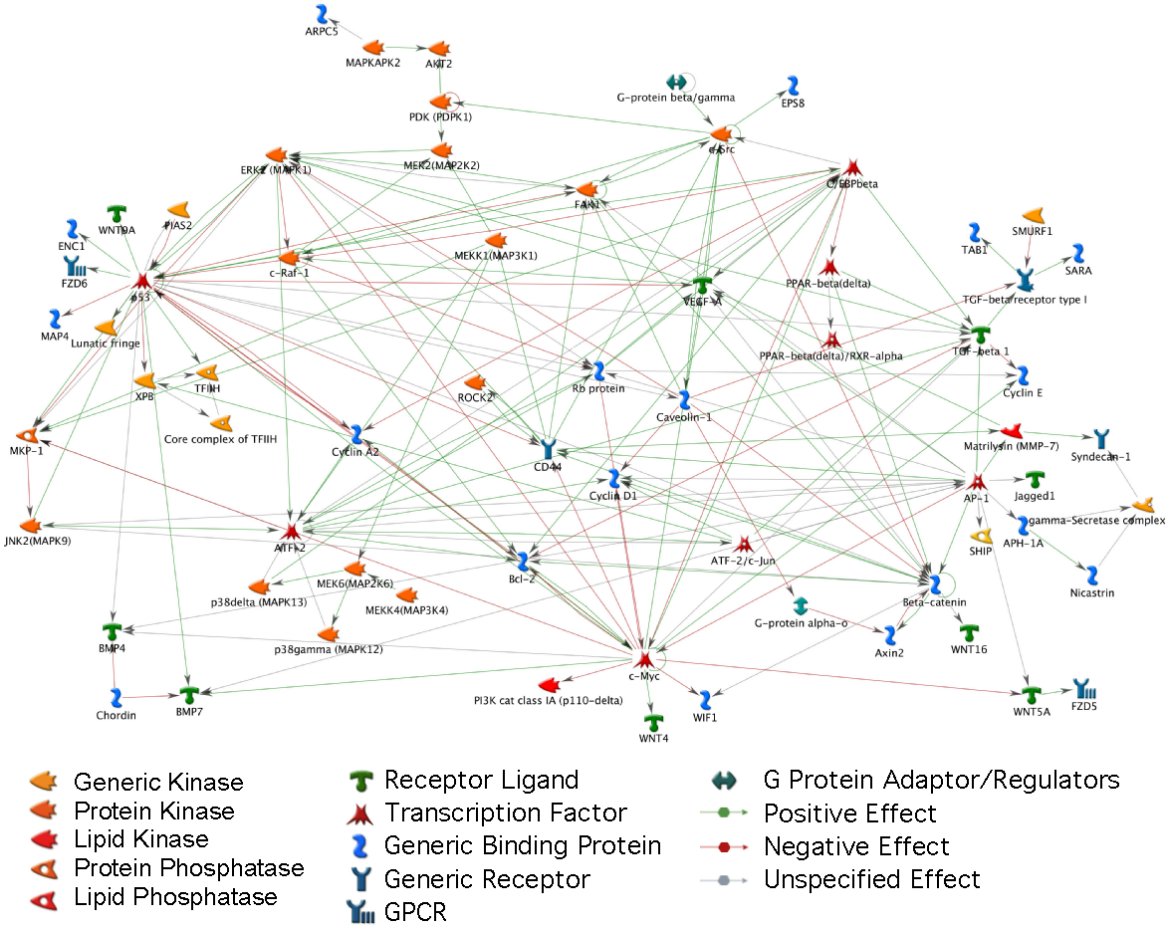


Fig. 9: Network of selected genes that show different expression patterns in mouse and rat in the comparison blastocyst versus morula. The graph has been created with GeneGo network editor tool (From Casanova et al, submitted Manuscript March 2011).

The goal of this study, as mentioned before, was to highlight differences and similarities in the gene expression in the morula and the blastocyst from mouse and rat, in order to identify important genes involved in the maintenance of pluripotency in ESCs, and especially of rat ESCs.
With the microarray analysis reported in the submitted manuscript, only the first part of the original goal is achieved. Actually, we were able to highlight differential regulation of important factors between the two species. However, these data represent only a preliminary analysis. In order to achieve the second goal of this project, namely increase the knowledge about the regulation of pluripotency in rat ESCs, a list of candidate genes have to be selected out of the obtained data.

The interesting genes, which might encode for important factors involved in the stabilization and maintenance of pluripotency in the rat ESCs, should be selected on the basis of different criteria:

- Their expression in the morula and in the blastocyst of the rat. Like for Nanog or for Pramel7, genes exclusively expressed in the inner part of the morula and in the ICM cells of the blastocyst represent good candidates for a further analysis in ESCs.
- The expression *in vivo* should be compared to the expression of the homolog in the mouse embryos. The expression at the RNA level can be drastically different or might be not, therefore an analysis on the protein level could be of advantage for clarifying differential regulations.
- The present knowledge about the function of the selected genes should be critically analyzed. The interactions of the selected genes with important pathways should be considered as well (see Fig. 9).
- Finally, of course the expression of the newly selected genes should be confirmed in the rat ESCs, and maybe, compared to the one observed in the mouse ESCs. In this case, the analysis of the function of the genes can be investigated by using the classical gain or loss of function analyses.

The high throughput technologies is a constantly developing field, therefore gene chips containing the sequences of recently identified or mapped genes are regularly generated. In our study, in the case of the rat gene expression analysis, we noted that many genes were not mapped on the rat chip, excluding therefore a complete cross-species comparison. Some important genes might therefore not be present in the data obtained. An example of not mapped genes on the rat chip (but present on the mouse chip) is Pramel7.
Nowadays, newly developed rat chips contain the sequences of Pramel7 and other newly mapped genes in the rat genome. Thus, these new chips could reveal the expression changes of a broader range of genes.

In conclusion, this study represents a good starting point for further analysis aimed at the identification of new factors related to pluripotency in both species.

3. Outlook

Although a number of valuable advances towards understanding the molecular mechanisms controlling and regulating pluripotency have been made, still many questions remain open.
ESC lines that keep their pluripotency after transfection and selection procedures are essential for the introduction of selected targeted mutations into the germ-line of the model animals. Thus, the identification of new genes able to stabilize ESCs would represent an important step for the stem cell research.

Concerning the first study reported in this Thesis, the identification of *Pramel7* as a crucial factor in ESCs, represents a start for further analyses, not only focused on the role of Pramel7 but maybe also extended to other members of the Pramel family. Seen the selected temporo-spatial expression of the Pramel genes, elucidating their molecular functions in the preimplantation development would give important information about the regulation of developmental processes. The members of the Pramel family are highly conserved through the evolution; therefore an investigation and comparison of their expression and functions in the mouse and in the rat could for instance explain why the preimplantation embryo development is shifted in the two species.

The results reported for Pramel7 in ESCs revealed that this gene plays a critical function in murine ESCs. Thus, the characterization of Pramel7 in rat ESCs would certainly be a next fundamental step to perform, in order to analyze if it could stabilize derivation and maintenance of rat cells. Preliminary data lead to assume a possible implication of Pramel7 in epigenetic processes. Since epigenetics is the power which drives a large part of the reprogramming mechanisms, a careful analysis of Pramel7 in the context of reprogramming (iPSCs) would give important information about its function.
Of course, all the above-reported analyses could be extended to other members of the Pramel family, which might play important functions in the determination and generation of pluripotent cells. Understanding these mechanisms is essential because ESCs and iPSCs hold great promise for the therapeutic treatment of human diseases.

The interaction of Pramel7 with Uhrf1 opens a number of questions regarding the molecular function of these proteins in ESCs. Answering these questions rendered indispensable the generation of antibodies against Pramel7, which are at the moment tested. The recently availability of Pramel7 antibodies represents another usable tool for the characterization of this gene.

The advent of the modern high throughput technologies will open new possibilities in understanding the mechanisms ruling cell fate. All this will lead to the discovery not only of new mechanisms and key players determining pluripotency, but also will allow expanding the knowledge how to drive controlled differentiation of pluripotent cells towards pure populations of precursors and terminally differentiated cells, by identifying the right signals and culture environments.

The genome wide cross species analysis that we performed comparing the mouse and the rat morula and blastocyst, will allow the identification of new factors implicated in differentiation and pluripotency processes. The selected pool of genes should then be analyzed *in vitro*, in murine and rat ESCs. The generation of transgenic mouse and rat ESCs overexpressing those genes will answer the question about their involvement in the maintenance of pluripotency. Moreover, some of these new candidate genes could play a critical role in the stabilization of ESCs in both the species. The functional characterization of these genes might dramatically improve the establishment of authentic ESCs and iPSCs from different strains and from different species, in particular the rat.

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Annex 1

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Expression profiling in transgenic FVB/N embryonic stem cells overexpressing STAT3

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Abstract

Background: The transcription factor STAT3 is a downstream target of the LIF signalling cascade. LIF signalling or activation is sufficient to maintain embryonic stem (ES) cells in an undifferentiated and pluripotent state. To further investigate the importance of STAT3 in the establishment of ES cells we have in a first step derived stable pluripotent embryonic stem cells from transgenic FVB mice expressing a conditional tamoxifen dependent STAT3-MER fusion protein. In a second step, STAT3-MER overexpressing cells were used to identify STAT3 pathway-related genes by expression profiling in order to identify new key-players involved in maintenance of pluripotency in ES cells.

Results: Transgenic STAT3-MER blastocysts yielded pluripotent germline-competent ES cells at a high frequency in the absence of LIF when established in tamoxifen-containing medium. Expression profiling of tamoxifen-induced transgenic FVB ES cell lines revealed a set of 26 genes that were markedly up- or down-regulated when compared with wild type cells. The expression of four of the up-regulated genes (Hexokinase II, Lefty2, Pramel7, PP1rs15B) was shown to be restricted to the inner cell mass (ICM) of the blastocysts. These differentially expressed genes represent potential candidates for the maintenance of pluripotency of ES cells. We finally overexpressed two candidate genes, Pem/Rhox5 and Pramel7, in ES cells and demonstrated that their overexpression is sufficient for the maintenance of expression of ES cell markers as well as of the typical morphology of pluripotent ES cells in absence of LIF.

Conclusion: Overexpression of STAT3-MER in the inner cell mass of blastocyst facilitates the establishment of ES cells and induces the upregulation of potential candidate genes involved in the maintenance of pluripotency. Two of them, Pem/Rhox5 and Pramel7, when overexpressed in ES cells are able to maintain the embryonic stem cells in a pluripotent state in a LIF independent manner as STAT3 or Nanog.

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Background

ES cell lines that maintain their pluripotency after transfection and selection procedures are essential for the introduction of selected targeted mutations into the mouse germ-line. Pluripotent ES cells are established *in vitro* from the inner cell mass (ICM) cells of explanted blastocyst-stage embryos [1-3]. Murine ES cells are maintained in a pluripotent state by co-culturing with mitotically-inactivated feeder cells, such as embryonic fibroblasts, and/or the addition of leukaemia inhibitory factor (LIF: [4,5]). These ES cells can be maintained indefinitely in the presence of LIF, and express markers of the undifferentiated and pluripotent state, including the POU-domain transcription factor OCT-3/4 (POU5F1), a factor that is essential for the development of the ICM (reviewed by [6]; [7]). Upon removal of LIF, the cells rapidly lose self-renewal capacity and differentiate into a variety of cell types. LIF belongs to the Interleukin-6 family of cytokines and the members of this family have diverse effects on a variety of cell types [8]. The shared usage of signal transducers (i.e. gp130) in the multichain cytokine receptor complexes clearly explains the functional redundancies of these cytokines (reviewed by [9]).

The pathway by which LIF signalling acts to promote ES cell self-renewal has been partially well studied (reviewed by [10]). LIF signals via heterodimerization of the two class I cytokine receptors, the low affinity LIF receptor (LIFR) and the common subunit, gp130. The cytoplasmic domain of gp130 contains several tyrosinase residues that are phosphorylated by associated JAK (Janus kinase) kinases after ligand-stimulated dimerization. Four of these phosphorylated tyrosines have been identified as putative interaction sites with the SH2 (Src homology 2) domain of the transcription factor STAT3 (signal transducer and activator of transcription; [11]). Stimulation of gp130 signalling in ES cells also phosphorylates SHP-2 (SH2-domain-containing tyrosine phosphatase) and leads to activation of the mitogen-activated protein (MAP) kinases ERK1 and ERK2 [12]. Inhibition of the SHP-2/RAS/ERK pathway promotes self-renewal and suppresses differentiation and treatment of mouse ES cells with the MAPK-inhibitor PD098059 [13] was shown to enhance self-renewal [14].

Matsuda et al. (1999) have shown that activation of the STAT3 transcription factor is sufficient to maintain mouse ES cells in an undifferentiated state in the absence of LIF [15]: An inducible transgene construct encoding the entire STAT3 coding region fused to the mutated ligand-binding domain of the estrogen receptor (STAT3-MER) was introduced into ES cells. ES cells expressing the STAT3-MER fusion protein maintained their undifferentiated state in the presence of OHT and in the absence of LIF [15]. This study highlighted the importance of STAT3 pathway in maintenance of ES cell pluripotency *in vitro*. However, the *in vivo* relevance of the LIF pathway is to date still not clear; LIF expression can be detected in the trophectoderm (TE) of the blastocyst whereas LIF receptor is expressed in the ICM. However, neither LIF mutants [16] nor mutants of the receptors LIFR [17,18] and gp130 [19] result in any defects in the development of the ICM or early epiblast. Recent evidence suggests that the LIF pathway is necessary for survival of the mouse epiblast during diapause [20].

ES cell lines derived from different mouse strains exhibit variable degrees of LIF dependency as demonstrated in STAT3 gene targeting experiments by Raz et al. [21]. ES cells heterozygous for a STAT3 mutation could only be established from E14 cells (129P2/OlaHsd; [22]). Targeted clones from other cell lines were invariably trisomic for chromosome 11 that carries the STAT3 locus, and thus retained normal levels of activated STAT3.

To date it is unclear if higher amounts of STAT3 in the inner cell mass of blastocyst support the survival and derivation of pluripotent ES cells, especially in so called non-permissive mouse strains like FVB/N. The inbred mouse strain FVB/N is widely used for the generation of transgenic animals [23], however only one germline competent ES cell line has been reported [24]. We therefore, in a first step, generated FVB/N transgenic mice overexpressing a tamoxifen inducible STAT3 (STAT3-MER). Our data demonstrated that overexpression of STAT3 in the ICM of the blastocyst supports the establishment of ES cells in the FVB/N mouse strain. ES cell lines overexpressing STAT3-MER were germline-competent whereas the only WT line that we could establish was not germline-competent.

Recent studies have begun to identify key players involved in the intracellular signal transduction pathways regulating stem cell renewal and proliferation. Several transcription factors including the OCT-3/4 have been shown to be essential to maintain pluripotency in the ICM, but none had been shown to function independently of the LIF pathway with exception of the newly identified homeobox transcription factor Nanog, that directs pluripotency in mouse ICM and mouse ES cells and functions independently from LIF dependent STAT3 activation [25,26]. Nanog is detected in the ICM and early germ cells, as well as in the ES and embryonic carcinoma (EC) cell lines derived from these stages [25]. Overexpression of Nanog relieves mouse ES cells cultured without feeder cells in the presence of serum from dependence on LIF stimulation for self-renewal whereas Nanog-deficient mouse ES cells loose pluripotency and differentiate into extra embryonic endoderm lineages [26].

We have further focused our study on the STAT3 pathway in order to elucidate the differences between WT and

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STAT3 overexpressing embryonic stem cells. We performed microarray-analysis comparing WT and STAT3-MER overexpressing FVB/N ES cells and identified a pool of genes that were differentially expressed. From the microarray dataset, we screened for potential candidates of pluripotency by their expression pattern in the early preimplantation embryo. Among these, we confirmed Pem/Rhox5 and Pramel7 as regulators of pluripotency using functional studies: ES cells overexpressing Pem/Rhox5 and Pramel7 were able to maintain typical pluripotent ES cell morphology in the absence of LIF, as well as the characteristic pluripotency-related markers SSEA-1 and Oct4 in a similar extent as Nanog which was used as a positive control. This clearly demonstrates that these two STAT3-pathway related genes are involved in the maintenance of pluripotency.

Results

Generation of transgenic mice overexpressing STAT3-MER

We produced FVB/N transgenic mice overexpressing a fusion protein composed of the entire coding region of mouse STAT3 and the modified ligand-binding domain (G525R) of the mouse estrogen receptor [15]. The modified ligand-binding domain binds the synthetic steroid ligand 4-hydroxytamoxifen (4OHT) but not 17β-estradiol [30]. The expression of the transgene is driven by a chicken β-actin promoter and therefore is expected to be ubiquitously expressed. After injection of the construct into the pronucleus of fertilized FVB/N eggs we obtained seven positive founder animals. When crossed with wild-type FVB/N partners, six of them showed germline transmission. A multiple tissue analysis was performed, by western blotting, in order to define animals exhibiting ubiquitous expression (data not shown). Two of highest STAT3-MER expressing lines (Tg741 and Tg743) were selected for further experiments. Both transgenic lines contained a single integration of the transgenic cluster and were maintained in a hemizygous state by constant breeding with WT FVB/N mice.

Overexpression of inducible active STAT3-MER enables the establishment of germline competent ES cells from FVB/N blastocysts

In order to test if the overexpression of STAT3-MER would allow the establishment of germline competent ES cells from the FVB/N mouse strain, morulae were flushed from the uterotubal junction 3 days after mating and cultured overnight in M16 medium (Sigma). Fully expanded blastocysts were transferred onto MEF as described in "Methods". Full-grown ICMs were picked from the outgrown TE and dissociated mechanically into groups of cells and these aggregates reseeded onto embryonic feeder fibroblasts. 3–4 days later, compact stem cell colonies could be identified. Single colonies were dissociated as described above and reseeded. Non-differentiating clonal lines were further passaged and split after 2–3 generations for further characterization. Embryos were cultivated in medium containing either LIF or OHT. We were able to establish both wild type and transgenic ES cell lines. However, even if ICMs from WT embryos were able to outgrowth from the TE in presence of OHT it was impossible to generate ES cell colonies during the further steps of cultivation and WT ES cells were only obtained when LIF was present in the medium (Table 1). When using OHT-supplemented medium without LIF 43–71% of the embryos from both Tg741 and Tg743 transgenic lines yielded ES cell lines, all of which were transgenic (Table 1). Because the mice used were hemizygous for the transgene, and therefore only 50% of the embryos is expected to be transgenic, it is fair to assume that we were able to derive ES cells from virtually all the transgenic embryos. These results strongly indicate a supportive effect of active STAT3-MER on the maintenance of pluripotent ES cells. The newly established ES cell lines overexpressing STAT3-MER were culti-

Table 1: Establishment of FVB/N ES cells

Founder Line	No Of embryos	Medium Supplements	No of ICM picked	No of stem cell colonies	Cell lines	Transgenic
WT	15	LIF	8 (53%)*	5 (62%)**	5	-
	15	OHT	4 (27%)*	0 (0%)**	-	-
741	28	OHT	23 (82%)*	10 (43 %)**	7	7 (100 %)#
743	28	OHT	14 (50%)*	10 (71 %)**	10	10 (100 %)#
741-F1	26	OHT	18 (69%)*	8 (44 %)**	8	8 (100 %)#

* percent ICM per plated embryo; ** percent colonies per picked ICM

percent transgenic per cell lines

WT FVB/N or hemizygous FVB/N males carrying the STAT3-MER transgene (lines 741 and 743) were mated with WT FVB/N females. Blastocysts were cultivated either in presence of LIF or OHT. Outgrown ICM's were picked and cultivated till ES cell colonies were visible. Cells were tested for the presence of the transgene by PCR analysis. As expected it was possible to establish WT ES cells only in presence of LIF but not when OHT alone was added to the culture. Furthermore in presence of OHT both transgenic lines 741 and 743 generated ES cell colonies that were carrying the transgene. Because theoretically only 50% of the blastocysts were expected to be transgene it is to assume that the establishment frequency was almost 100%. After generation of germline competent chimera with the newly established 741 cell line hemizygous transgenic F1 animals were generated and mated with WT FVB/N females. In this breeding the establishment efficiency in presence of OHT was similar to one observed in the parental blastocysts indicating that the STAT3-MER induced stabilization is kept also in the F1 generation.

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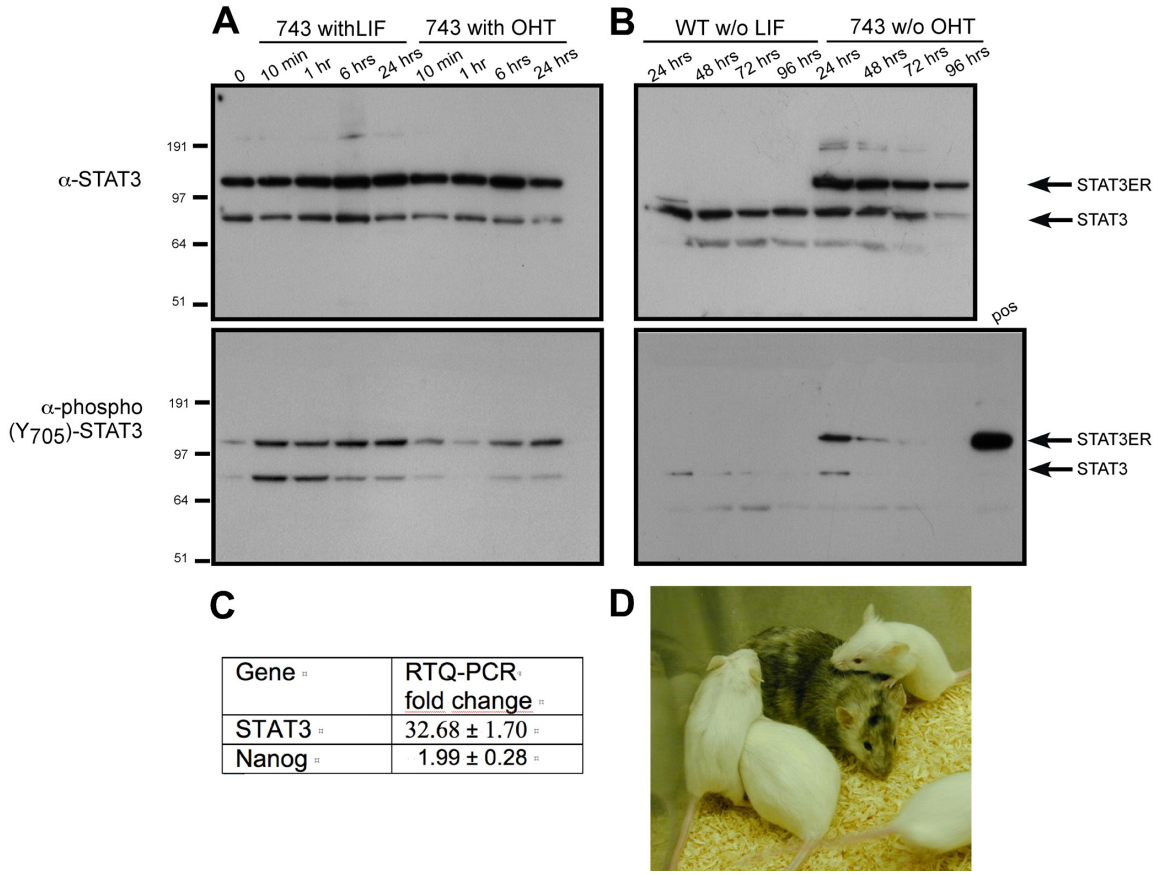

Gene	RTQ-PCR fold change
STAT3	32.68 ± 1.70
Nanog	1.99 ± 0.28

Figure 1

Characterization of the 743 transgenic ES cell line. **A.** Phosphorylation/dephosphorylation analysis of Tyr705 in endogenous and transgenic STAT3. After 24 hours of LIF and OHT deprivation cells were cultivated till their homogenization in presence of either LIF or OHT. During LIF or OHT deprivation no changes in the protein expression levels could be detected, but after 24 hrs the Tyr705 residue of both transgenic and WT STAT3 was completely dephosphorylated. 10 minutes after addition of LIF the tyr705 residue of both STAT3 was phosphorylated whereas after addition of OHT complete phosphorylation was obtained only after 6 hrs. **B.** Dephosphorylation of Tyr705 was analyzed by eliminating LIF or OHT from respectively WT or 743 cells. Kinetics for the dephosphorylation were slower then for the phosphorylation, only after 48 hrs dephosphorylation of WT Tyr705 was complete whereas complete dephosphorylation of STAT3-MER occurred only after 72 hrs. **C.** Nanog and STAT3 expression levels were tested by RTQ-PCR. Values are normalized with β-actin and the values indicate fold changes compared to the WT. Both WT and 743 ES cell lines expressed Nanog and the transgenic 743 ES cells have an increased Nanog expression compared to WT. **D.** After injection of 743 in C57BL/6 host blastocysts a 50–60% chimera was generated. Littermates from the crossing of the chimera with a WT FVB/N female generated white littermates, 50% of which were hemizygous for the transgene, indicating germline competence of the 743 cell line.

vated further only in presence of OHT without LIF. In order to confirm the pluripotency of these ES cells, karyotypically normal male cells from both transgenic lines (Tg741 and Tg743) were injected into C57BL/6 host blastocysts. Chimeric males were identified by the absence of eye (pink) and coat (albino) pigmentation and mated to wildtype FVB/N females. Germline transmission of the FVB/N ES cell genome resulted in albino offspring (Figure 1D). None of the 5 FVB WT ES cell lines was able to produce chimeras when injected into C57BL/6 blastocysts (data not shown). To confirm that overexpression of active STAT3 supports the survival and derivation of pluripotent ES cells also in the F1 generation, transgenic germline F1 offspring from the line Tg741 were mated to

wildtype animals. Blastocyst stage embryos were isolated and cultivated as previously described, if cultivated in presence of OHT stem cell lines could be established from 44% of the embryos, all lines being transgenic (Table 1).

Characterization of the newly established FVB/N ES cells overexpressing STAT3-MER

The expression level of STAT3-MER in the ES clones obtained from the line 743, was tested by western blot (see Figure 1A and 1B). Upon LIF stimulation STAT3 is phosphorylated on the tyrosine residue (Y705), dimerizes and can bind DNA [31,32]. In order to test if OHT is able to induce STAT3-MER phosphorylation FVB/N ES cells expressing STAT3-MER were first deprived of LIF or OHT for 24 hrs, after this time the Tyr705 residues of both the endogenous STAT3 and STAT3-MER were completely dephosphorylated. After the 24 hrs deprivation cells were stimulated either with LIF or OHT for 10 minutes up to 24 hours and further cultivated in presence of LIF or OHT till their homogenization. Cell extracts were separated by SDS-PAGE, blotted and probed with anti-STAT3 and anti-phospho (Y705) antibodies. LIF stimulation induced tyrosine phosphorylation of both endogenous STAT3 and STAT3-MER (Figure 1A). As previously observed [15], endogenous STAT3 was rapidly phosphorylated whereas phosphorylation kinetics of STAT3-MER were slower. In ES cells derived from Tg743 stimulation with OHT resulted in a strong tyrosine phosphorylation of STAT3-MER, but only a limited phosphorylation could be detected for endogenous STAT3 (Figure 1A). During the 24 hours of induction with either LIF or OHT expression of Oct4 was confirmed (data not shown). Dephosphorylation kinetic of Tyr705 was also analyzed by eliminating LIF or OHT from the medium of respectively WT or 743 cells. Kinetics for the dephosphorylation were slower then for the phosphorylation, only after 48 hrs dephosphorylation of WT Tyr705 was complete whereas complete dephosphorylation of STAT3-MER occurred only after 72 hrs. Dose dependence for dephosphorylation could also be observed. In ES cells derived from the Tg743 line, expressing higher amounts of STAT3-MER, dephosphorylation was slower compared to cells derived from the lower expressing Tg747 line (data not shown).

ES cells overexpressing STAT3-MER express the typical ES-cell markers

ES cells, as well as cells of the ICM of mouse blastocysts, express a panel of markers that are used to characterize undifferentiated, pluripotent embryonic cells, between them Nanog, alkaline phosphatase, OCT-3/4 and SSEA-1 are the most typically used. Nanog expression was tested by RTQ-PCR; both WT and 743 ES cells expressed Nanog and a light overexpression could be detected in the 743 cells if compared with WT cells after normalization with the housekeeping gene β-actin (Figure 1C). The expression of the transcriptional factor OCT-3/4 and the surface marker SSEA-1 was tested by immunohistochemistry (Figure 2A); WT FVB as well as both transgenic lines 743 and 741 expressed both markers. Furthermore, all three cell lines expressed the marker alkaline phosphatase (Figure 2B). In all cases the expression was restricted to the ES cells and no signal could be detected in the inactivated fibroblast used as feeder cells.

Microarray analysis

Even though it was possible to establish WT FVB ES cells in presence of LIF and these cells express the typical markers for ES cells they were not able to generate chimeric mice. This suggests that overexpression of STAT3-MER could increase the level of pluripotency in FVB ES cells. In order to understand the difference between the WT cells and the germline competent 743 cells we decided to compare the gene expression profiles of both lines. We compared three independently cultivated dishes of WT FVB cells cultivated in the presence of LIF with three independently cultivated dishes of the transgenic 743 cells overexpressing STAT3-MER cultivated in the presence of OHT. Total RNA was isolated and an expression analysis was performed by hybridizing U74v2 Affymetrix chips containing probes covering the complete mouse transcriptome (36'000 transcripts). Analysis was performed with dCHIP by using both the PM/MM (perfect match/mismatch) difference model and the PM (perfect match) only model in order to compare the results [33]. Genes showing expression changes higher then 1.5 fold were considered. As control, we first confirmed that the overexpression of STAT3-MER in the 743 line was 33 times higher than in the WT cells. We further identified a set of 26 differentially regulated genes, 13 were upregulated (Table 2) whereas 13 were downregulated (Table 3).

In situ Hybridization

In a first step we analyzed which of the differentially expressed genes had already previously been described in the literature to be expressed during preimplantation mouse development and therefore potentially play a role in maintenance of pluripotency. Eight genes out of the 26 identified were considered as candidates to have a potential function in determination and maintenance of pluripotency in ES cells. For these genes *in situ* hybridization was performed in order to define the regions of preimplantation embryos in which they were expressed. The temporo-spatial expression was analyzed by whole mount *in situ* hybridization of morulae and blastocysts. Four genes, Pramel7, Lefty2, Protein Phosphatase 1 regulatory subunit 15B and hexokinase II were expressed only in the central part of the morula and in the ICM of the blastocyst (Figure 3A). The other five genes, Pramel6, Eif2s2, Pem/Rhox5, Dppa3 and Skp2 were found to be expressed in all cells of the morula and blastocysts (Figure 3A). Because

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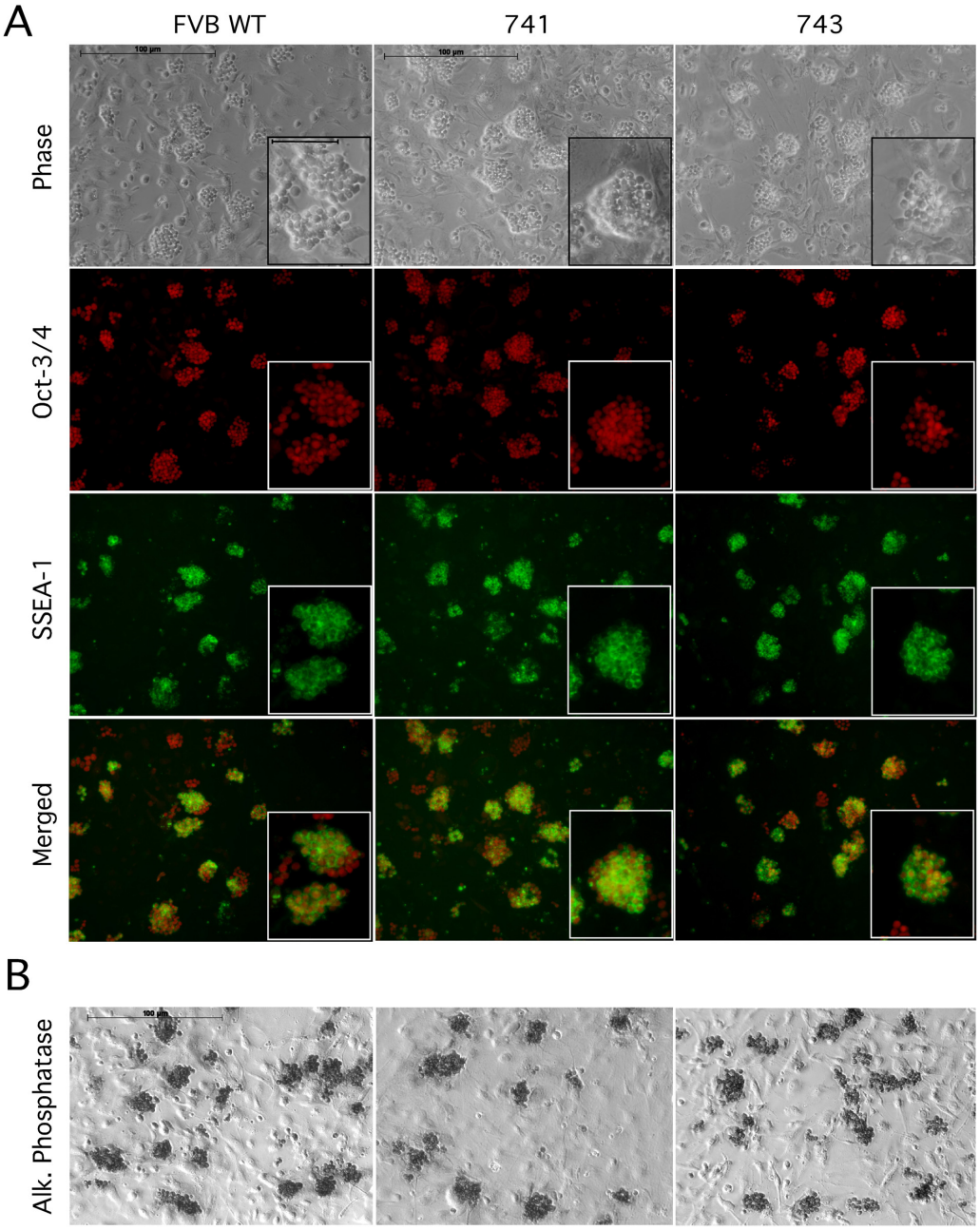

Figure 2
Immunohistochemical analysis of WT, 741 and 743 ES cell lines. All three cell lines express the nuclear marker OCT-3/4 and the surface marker SSEA-1 (A) and alkaline phosphatase (B). The expression of all markers was restricted to ES cells. Mouse fibroblast used as feeder cells are negative for OCT-3/4, SSEA-1 and alkaline phosphatase. Scale bar in both large and small panels: 100 μm.

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Table 2: Upregulated genes

Probe set	Identifier	RefSeq	Chr	MM/PM fold change	MM/PM p-value (t-test)	PM fold change	PM p-value (t-test)	RTQ-PCR fold change
98524_f_at	**X-linked myotubular myopathy gene 1 (Mtm1)**	NM_019926	X	1.87	0.018906	1.91	0.017234	n.d.
94375_at	**Hexokinase 2 (Hk2)**	NM_013820	6	1.6	0.018958	1.61	0.015899	1.79 ± 0.11
95545_at	**Insulin-like growth factor 1A (Igf1)**	NM_010512	10	2.34	0.003079	1.6	0.007358	n.d.
98059_s_at	**lamin A (Lmna)**	NM_001002011	3	1.58	0.070634	1.47	0.035881	n.d.
95562_at	**Nuclear domain 10 protein 52 (Ndp52)**	NM_029755	11	2.66	0.065377	2.08	0.057843	6.72 ± 0.67
101368_at	**Reproductive homeobox 5 (Rhox5/Pem)**	NM_008818	X	1.68	0.029649	1.82	0.013529	3.64 ± 0.3
103470_at	**Pramel6**	NM_178249	2	6.18	0.02356	4.14	0.027034	10.0 ± 0.37
93245_at	**Pramel7**	NM_178250	2	3.51	0.051948	2.77	0.052927	8.51 ± 0.92
95609_at	**Protein phosphatase 1, regulatory (inhibitor) subunit 15b (Ppp1r15b)**	NM_133819	1	1.6	0.024109	1.5	0.022545	1.56 ± 0.12
92232_at	**suppressor of cytokine signaling 3 (Socs3)**	NM_007707	11	1.75	0.053978	1.56	0.015837	2.34 ± 0.35
101449_at	**tripartite motif-containing 41 (Trim41)**	NM_145377	11	-	-	4.11	0.000819	1.07 ± 0.17
160365_at	**Eukaryotic translation initiation factor 2, subunit 2 beta (Eif2s2)**	NM_026030	2	1.98	0.040383	1.96	0.042002	1.80 ± 0.09
160420_r_at	**tubulin, alpha 3A (Tuba3a)**	NM_009446	6	2.07	0.080906	1.93	0.078476	n.d.

Genes upregulated after STAT3-MER activation through OHT. Microarray values are given as fold changes ratios relative to normalization derived from dCHIP. 13 genes were found to be significantly upregulated when compared with WT FVB/N cells cultivated in presence of LIF. Only genes with a 1.5× fold increase were considered as significant. The mRNA expression levels (mean ± standard deviation, n = 3) of relative to FVB WT control cells were validated by real time PCR analysis, RTQ-PCR values are ratios relative to β-actin (housekeeper gene) expression. For all selected genes measured, RT-PCR corroborated the rank order of magnitude of expression measured on the microarrays. Calculations were performed with both the PM/MM (perfect match/mismatch) difference model and the PM (perfect match) only model in order to compare the results.

the Pramel7 expression was restricted to the central part of the morula and in the ICM of the blastocyst, a more exact analysis of the preimplantation stages was performed (Figure 3B). Expression of Pramel7 starts at the compacted morula stage, no expression could be detected in earlier developmental stages indicating that this gene fulfils the requirements for being a potential candidate involved in maintenance of pluripotency. A similar expression pattern can be observed for Nanog [25].

Overexpression of Pem/Rhox5 and Pramel7 is sufficient for maintenance of ES cells in the absence of LIF

In order to test if Pramel7 is able to maintain pluripotency without direct activation of the STAT3 cascade through LIF the full-length cDNA of Pramel7 was inserted in the pfloxedNanog vector (see Chambers et al., 2003) instead of the cDNA of Nanog, and the vector was electroporated in E14 ES cells. In parallel the full-length cDNA of Pem/Rhox5 was also cloned in the same way into the pfloxedNanog vector. Pem/Rhox5 was previously described to play a role in maintenance of pluripotency, but it is not yet known if it is transcriptionally regulated through STAT3. As a control for the experiments the pfloxedNanog vector itself was also electroporated in E14 cells. All electroporated cells were selected with puromycin and resistant colonies were picked and expanded. After testing for the presence of the vectors by PCR the positive clones were analyzed by real time PCR and the clones with the strongest expression were used for further experiments. In order to test for the capacity of maintaining pluripotency in absence of LIF, the cells were cultivated for 8 days without addition of LIF to the medium. After 8 days in culture IHC was performed in order to detect the expression of OCT-3/4, SSEA-1 and alkaline phosphatase (Figure 4). E14 WT ES cells started after 4 days to differentiate and showed the typical flattened morphology of differentiating cells (data not shown), after 8 days the cells were completely differentiated and no longer expressed OCT-3/4 and SSEA-1. Nanog overexpressing cells as expected maintained their pluripotent state also in absence of LIF. Both Pramel7 and Pem/Rhox5 overexpressing clones showed a similar behaviour as Nanog overexpressing cells. The colonies maintained the typical round shaped morphology and expression of OCT-3/4 and SSEA-1 was present indicating that these two genes were able to maintain pluripotency also in absence of LIF.

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Table 3: Downregulated genes

Probe set	Identifier	RefSeq	Chr	MM/PM fold change	MM/PM p-value (t-test)	PM fold change	PM p-value (t-test)	RTQ-PCR fold change
97283_at	**developmental pluripotency-associated 3 (Dppa3)**	NM_139218	6	-2.16	0.10982	-2.81	0.047749	-11.4 ± 1.51
133948_at	**Eukaryotic translation initiation factor 2C 4 (Eif2c4)**	AI504948	17	-1.53	0.044727	-1.46	0.018363	n.d.
134566_at	**Left-right determination factor 2 (Lefty2)**	NM_177099	1	-2.7	0.029827	-2.32	0.025196	-2.35 ± 0.23
103389_at	**aminoadipate-semialdehyde synthase (Aass)**	NM_013930	6	-2.07	0.077262	-1.71	0.100000	n.d.
167617_r_at	**transmembrane protein 109 (Tmem 109)**	NM_134142	19	-1.89	0.036619	-1.73	0.042999	n.d.
166131_at	**enabled homolog (Enah)**	NM_001083120	1	-1.55	0.008738	-1.46	0.016412	n.d.
138014_at	**nucleoporin 153 (Nup 153)**	NM_175749	13	-1.55	0.012282	-1.49	0.011324	n.d.
167824_f_at	**S-phase kinase-associated protein 2 (p45/Skp2)**	NM_013787	15	-1.74	0.054672	-1.62	0.073491	-1.27 ± 0.03
100499_at	**syntaxin 3 (Stx3)**	NM_001025307	19	-2.09	0.033994	-2.03	0.036655	n.d.
166600_at	**KH domain containing, RNA binding, signal transduction associated 3 (Khdrbs3)**	NM_010158	15	-1.66	0.027322	-1.52	0.035974	n.d.
93568_i_at 93569_f_at	**RIKEN cDNA 2610042L04 gene**	BC096548.1	14	-3.29	0.069938	-2.94	0.070848	n.d.
161004_at	**RIKEN cDNA 1700097N02 gene**	XM_001479022	17	-2.12	0.100000	-2.24	0.002726	n.d.
167843_f_at	**similar to RIKEN cDNA 1110051B16 gene**	XM_001472554	14	-2.71	0.004646	-1.81	0.003623	n.d.

Genes downregulated after STAT3-MER activation through OHT. 13 genes were found to be significantly downregulated when compared with WT FVB/N cells cultivated in presence of LIF. Only genes with a 1.5× fold decrease were considered as significant. The expression levels of selected genes were confirmed by real time PCR analysis. Calculations were performed with both the PM/MM (perfect match/mismatch) difference model and the PM (perfect match) only model in order to compare the results.

Discussion

ES cell lines derived from different mouse strains exhibit variable degrees of LIF dependency and to date it was unclear if higher amounts of STAT3 in the inner cell mass of blastocyst could support the survival and derivation of pluripotent ES cells in non-permissive mouse strains. Our work indicates that the activation of the STAT3 pathway during cultivation of blastocysts supports ICM outgrowth and clearly favors the establishment of new ES cell colonies in the so called non-permissive FVB mouse strain.

Even though we were able to establish WT FVB/N ES cells in the presence of LIF these cells were not fully pluripotent and were unable to generate chimeras. Only through the overexpression of STAT3-MER cells we were able to establish germline competent FVB/N ES cells. Furthermore, in presence of OHT both transgenic lines 741 and 743 generated ES cell colonies with a very high efficiency. Theoretically only 50% of the blastocysts were expected to be transgenic and this is also the establishment efficiency we obtained. It is therefore fair to assume that the establishment frequency was almost 100% (Table 1).

Interestingly no changes in the regulation of the classical marker for pluripotent ES cells could be detected between WT and 743 cells. Alkaline phosphatase, OCT-3/4, SSEA-1 were correctly expressed in both wildtype and transgenic ES cell lines (see Figure 2A and 2B). RTQ-PCR showed a slight upregulation of Nanog in the 743 ES cells (Figure 1C). If this upregulation is due to a direct or an indirect interaction with STAT3 has to be further analyzed. The different roles of the LIF pathways and of Nanog are yet not very clear. ES cell populations are heterogeneous and it is also known that Nanog is expressed discontinuously in pluripotent cells in the embryo and it is therefore to assume that Nanog and the LIF pathway interact to some extent in controlling the different events that regulate pluripotency and self-renewal. The recent findings of Chambers et al. (2007) corroborate this hypothesis. The authors demonstrated that Nanog expression was specifically required for both the formation of the ICM and of the germ cells, rather than for the housekeeping machinery of pluripotency embryonic, and that stem cells could self-renew indefinitely in the permanent absence of Nanog [34].

We were further interested in the identification of the molecular changes induced through STAT3-MER overexpression. We therefore decided to identify STAT3-pathway related genes by expression profiling. In general, there is great interest in identifying the signature of stemness by the constellation of genes that stem cells express. DNA microarray technology allows the discovery of a large number of genes that are thought to be the molecular signature of mouse ES cells. Recently, global transcription profiles of undifferentiated ES cells and blastocyst have been reported by several groups, most of these studies were comparing differentiated versus undifferentiated

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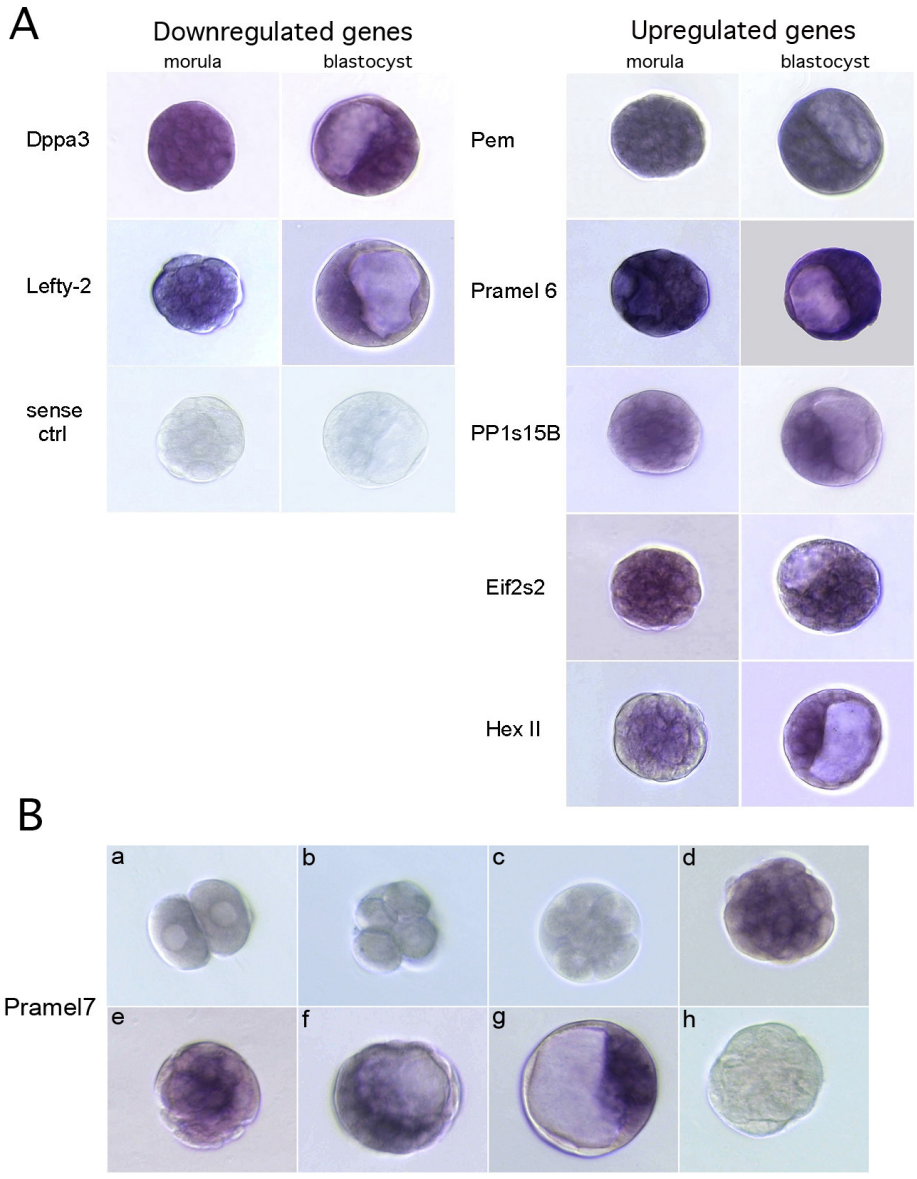

Figure 3
***In situ* hybridization of the eight selected genes**. **A.** Dppa3, Eif2s2, Pem/Rhox5 and Pramel6 exhibited a general expression in all cells of the morula and of the blastocyst. Expression of Hexokinase II, Lefty2 and PP1s15B was restricted to the cells of the inner part of the morula and to the ICM in the blastocyst. Sense probes in the same concentration of the antisense probes were used as negative controls for the hybridization. **B.** In situ hybridization with a Pramel7 antisense riboprobe of preimplantation embryos (a: two-cell embryo, b: four-cell embryo, c: eight-cell embryo; d, e: compacted morula, f, g: blastocyst, h: negative control with sense riboprobe). Magnification 40×.

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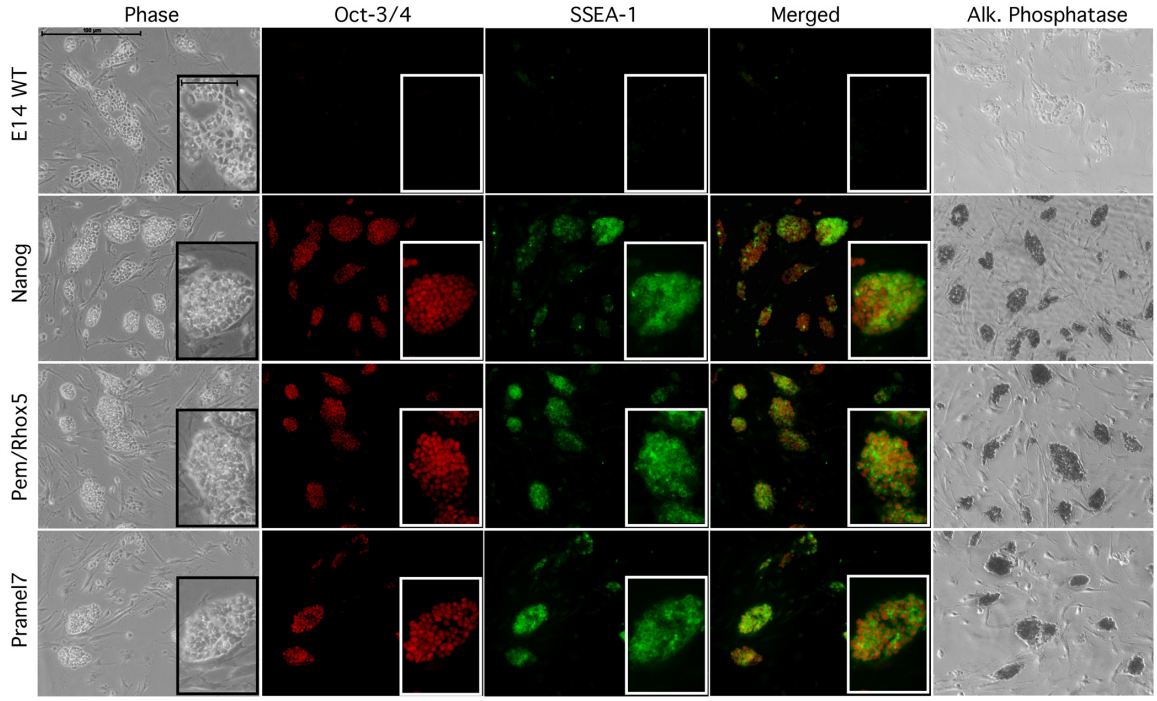

Figure 4
Immunohistochemical analysis of WT E14 ES cells and E14 overexpressing, Nanog, Pem and Pramel7. IHC analysis with OCT-3/4 and SSEA-1 and staining for alkaline phosphatase were performed after cultivation of the cells for 8 days without adding LIF to the medium. E14 ES cells differentiated under these conditions and lost the expression of OCT-3/4 and SSEA-1 and alkaline phosphatase. Both Pem/Rhox5 and Pramel7 overexpressing cells maintained the expression of the pluripotency markers. Nanog overexpressing cells, used as a control for the experiment, as expected maintained their pluripotency. Scale bar in both large and small panels: 100 μm.

cells [35-38]. In our study the FVB/N WT cells and the FVB/N cells overexpressing STAT3-MER are very similar, they just express different amounts of the transcription factor STAT3 and therefore we assumed that they would differentially regulate only a few genes. We compared gene expression changes between FVB/N ES cells overexpressing activated STAT3 cultivated in the presence of OHT and the absence of LIF and WT FVB cells cultivated in the presence of LIF by microarray analysis and identified a group of 26 genes that showed significant differential expression. From this list we preselected interesting genes by a careful literature and gene-expression databank analysis and identified which genes were characteristically expressed during the mouse preimplantation development.

These genes can be attributed to different categories according to their function: The first group contains regulatory members of the STAT3 pathway that are involved in the regulation of downstream events of the JAK/STAT cascade (like SOCS-3, protein phosphatase-1 regulatory subunit 15B and Eif2s2), the second group of genes is involved in the regulation of ES cell metabolism, whereas the third group contains genes that are involved in pluripotency maintenance and cell viability.

In the first group, among others, we found upregulation of SOCS-3 in STAT3-MER overexpressing cells. SOCS3 is a member of the suppressor of cytokine signalling (SOCS) family which has been implicated in the negative regulation of several pathways, in particular the JAK/STAT pathway, which can in turn induce SOCS expression and form a negative feedback circuit. The transcriptional upregulation of SOCS-3 confirms that the functional overexpression of STAT3-MER induces the activation of the classical LIF-dependent negative feedback mechanism (for a review see [39]). Previously Duval et al. (2000) showed that expression of SOCS-3, but not SOCS-1 and SOCS-2, was stimulated in ES cells in presence of LIF. The author further demonstrated that, uncontrolled overexpression

of SOCS-3 leads to repression of LIF-dependent transcription and severely reduces cell viability. This suggests that the disturbance of a well-balanced SOCS protein content has adverse effects on cell survival [40]. Since the FVB ES cells overexpressing STAT3-MER were viable and pluripotent, it is safe to assume that the SOCS-3 upregulation observed in presence of OHT is a modulatory reaction due to the overproduction of STAT3 in these cells. Through this compensatory mechanism the cells are able to maintain a properly activated LIF signalling cascade. It seems that the upregulation of SOCS-3 is a direct transcriptional activation mediated through STAT3 because the promoters of both mouse and rat SOCS-3 genes contain putative STAT1/STAT3 binding elements, which are necessary and sufficient for LIF-dependent activation of the SOCS-3 promoter activity in reporter assays [41,42].

We also found protein phosphatase 1 regulatory subunit 15B (PP1rs15B) and the elongation initiation factor 2 subunit 2 (eIF2s2) to be among the upregulated genes. PP1rs15B is a constitutively expressed inhibitory subunit of PP1, one of the major eukaryotic serine/threonine phosphatases. PP1rs15B dephosphorylates the α-subunit of eIF2 [43]. The microarray data confirm an upregulation of both PP1rs15B and eIF2s2 indicating that these proteins may be involved in the control of the STAT3 overexpression, whether these genes have a function in maintenance of pluripotency is still unclear.

In the second group we found hexokinase II to be increased in cells overexpressing STAT3, this confirms the importance of this isozyme for embryo viability and indicates that a correct energetic balance is extremely important in the late stages of preimplantation and at the beginning of postimplantation in the embryos.

The third group of differentially expressed genes comprises molecules involved in the maintenance of pluripotency and cell viability. Some of the identified genes were previously correlated with pluripotency [44] or with embryo viability in somatic nuclei derived cloned blastocysts [45].

Lefty2 is regulated by pathways such as Smad2/3 and WNT and by OCT-3/4, which support stemness. Lefty is also induced upon exit from the state of stemness, including forced in vitro differentiation and LIF withdrawal. When LIF is withdrawn, the expression of Lefty increases within 48 hours of cytokine withdrawal [37]. Similarly, retinoic acid that induces differentiation leads to increased expression of Lefty in mouse embryonic carcinoma cells [46]. Differentiation of stem cells to embryoid bodies also leads to increased expression of Lefty in vitro [47]. Therefore, Lefty might be important both to the stemness and differentiation events that follow the exit from this state.

Murine Pem/Rhox5 is an X-linked homeobox-containing gene [48,44], whose homeodomain shares important structural features with two other homeobox genes, which are expressed in extra embryonic lineages and during spermatogenesis [49,50]. The Pem/Rhox5 protein is expressed in the late morula stage, in TE and ICM of blastocyst and after implantation in extra embryonic tissues, in the parietal and visceral endoderm, but not in the primitive ectoderm derivatives. Pem/Rhox5 is also expressed in ES cells, in primordial germ cells and in teratocarcinoma cell lines [51]. Overexpression of Pem/Rhox5 had no phenotype in ES cells, but completely inhibited differentiation into the three primary cell lineages, when ES cells were cultured as embryoid bodies in suspension without LIF [52]. Two different models of action for Pem/Rhox5 are possible. Fan et al. (1999) suggested that Pem/Rhox5 first helps to maintain the undifferentiated cell state, and in a second step promotes a defined cell population of undifferentiated stem cells for differentiation into extra embryonic lineages [52]. Sasaki et al (1991) proposed an alternative in which Pem/Rhox5 directs early differentiation to specific lineages, but does maintain actively the undifferentiated state [44]. Our data are in line with previous studies and indicate that Pem/Rhox5 plays an important role in maintaining pluripotency of ES cells in absence of LIF (Figure 4)

Furthermore, overexpression of STAT3-MER induced differential expression of four genes (Dppa3, NDP52l1, Pramel6, Pramel7) that were identified as a set of OCT-3/4 related genes that were not correctly reactivated in somatic nuclei derived cloned embryos and therefore represent genes that are necessary for embryo viability [45]. Dppa3 (developmental pluripotency-associated 3; Stella; PGC7; Crg1) is preferentially expressed in primordial germ cells, oocytes and preimplantation embryos. In blastocysts, Dppa3 is expressed in TE and ICM and in the early postimplantation embryos Dppa3 expression disappears. The expression re-emerges when at day E7.5 the first primordial germ cells (PGCs) appear [53]. Dppa3 knockout mice are compromised in development; some embryos develop to the two or four cell stage, but fail to reach 8-cell stage [45,54]. Dppa3 was proposed by Sato et al. to play a role in germ line specification in mice by preventing nascent germ cell populations from a somatic cell fate and by retaining their pluripotency [53]. The embryonic function of NDP52l1 (Nuclear dot protein 52) is to date unclear but it is capable of forming dimers and contains leucine zipper motifs indicating a possible function in splicing processes [55].

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Pramel6 and Pramel7 (preferentially expressed antigen in melanoma like 6 and 7) are prevalently expressed in preimplantation embryos and embryonic pluripotent cells [45]. Our results confirm these expression patterns and clearly show that whereas Pramel6 is typically expressed in all cells of the morula and blastocyst, Pramel7 is expressed only in the inner part of the morula and in the ICM of the blastocyst. The function of the Pramel genes in embryonic development is unknown, but interestingly, PRAME inhibits retinoic-acid induced differentiation in mouse embryonic carcinoma F9 cells [56]. Recently Kaji et al (2006) showed that Pramel6 and Pramel7 expression is mediated by Mbd3, a component of the nucleosome remodelling and histone deacetylation (NuRD) complex [57]. Kaji et al. proposed that the Mbd3/NuRD-mediated silencing of Pramel6 and Pramel7 in ES cells offers an epigenetic environment in which Mbd3/NuRD is not absolutely required but facilitates differentiation. Furthermore the authors describe that Mbd3 deficiency leads to down regulation of Dppa3 in ES cells. Taken altogether, expression pattern analysis suggests that Dppa3, Pramel6 and Pramel7 are collaborating in deciding the fate of ES cells.

We were further interested in clarifying if some of the identified STAT3-pathway related genes could individually maintaining pluripotency in ES cells. Pem/Rhox5 and Pramel7 were chosen for this experiments: Pramel7 because of its characteristic expression pattern in the central part of the morula and in the inner cell mass of the blastocyst, an expression pattern which is very similar to the one observed for Nanog [25] and is typical for a gene involved in maintenance of pluripotency. Pem/Rhox5 because it was previously described in the literature as a gene blocking the differentiation capacity of the ICM and of ES cells [52,51]. Overexpression of Pramel7 and Pem/Rhox5 in ES cells and cultivation in absence of LIF, allowed the maintenance of pluripotency, as shown by IHC with the typical pluripotency related markers OCT-3/4 and SSEA-1 and with alkaline phosphatase staining, indicating the importance of these proteins in maintaining ES cells in a pluripotent state (Figure 4).

Conclusion

In summary the data presented here indicates that the overexpression of functional STAT3-MER in FVB/N blastocysts sustains and facilitates the establishment of germline competent ES cells in absence of LIF. Our findings open up the possibility of establishing germline competent ES cells from non-permissive mouse strains by manipulation of the STAT3 signal transduction pathway. Furthermore, gene expression analysis of these transgenic cells cultivated in presence of OHT showed that 26 genes were differentially expressed compared to WT cells cultivated in presence of LIF. By *in situ* hybridization analysis it was possible to identify four up-regulated genes (Hexokinase II, Lefty2, Pramel7, PP1rs15B) whose expression was restricted to the ICM of the blastocysts. Overexpression of two of the upregulated genes, Pem/Rhox5 and Pramel7, in E14 cells and cultivation in absence of LIF demonstrated that these two genes are able to maintain the embryonic stem cells in a pluripotent state without addition of LIF to the culture media.

Methods

Generation, Identification, and Maintenance of Transgenic Mice

The pCAGmusstat3ER plasmid containing the full length sequence of murine STAT3 cDNA fused to the ligand-binding domain of mouse estrogen receptor under the control of the chicken β-actin promoter [15] was propagated in *Escherichia coli* DH5α and the minigene was excised with NotI. The fragments were purified from a 1% agarose gel with a Qiaquick extraction kit (Qiagen, Basel, Switzerland) and processed as described. Nuclear injections into fertilized FVB/N oocytes were carried out by conventional methods [27,28]. Transgenic founder were identified by PCR using the β-actin promoter specific primer ggbactfor2: (5'-GGG TTC GGC TTC TGG CGT G-3') and a STAT3 specific primer mmSTAT3back2: (5'-CCA AGG TGC CAG GAA CTG CCG-3'). Two primers specific for the TAG-1 gene (TAG82B: 5'-ACA CGA AGT GAC GCC CAT CCG T-3'; TAG83F: 5'-GGA GGA GAG AGA CCC CGT GAA A-3') were used as a positive control for both wild-type and transgenic mice. ggbactfor2 and mmSTAT3back2 generate a 397 bp band whereas TAG82B and TAG83F generate a 300 bp product.

Cell culture

ES Cell Medium for FVB/N ES cells

KSR-KDMEM (Invitrogen) with 1000 units per ml human LIF, KSR-KDMEM w/o LIF and KSR-KDMEM w/o LIF with 1 μM 4-hydroxytamoxifen (OHT, Sigma).

ES Cell Medium for E14 ES cells

G-MEM (Sigma) containing 100 mM sodium pyruvate (GIBCO), 10%FBS, 50 mM β-mercaptoethanol and containing 10^7 U/ml ESGRO murine LIF (Chemikon Int.).

Embryonic feeder fibroblasts (MEF)

were derived from explanted day 14 foetuses of CD-1-MTKneo strain mice [29]. Prior to co-culturing with ES cells, confluent layers of MEF (passage 2) cells were treated with mitomycin C (10 μg/μl) for 2.5 hrs and extensively washed. Growth arrested fibroblasts were used as feeder cells for up to one week.

Embryo recovery, embryo culture, isolation of ES cell lines

Donor females of strains FVB/N (RCC Füllinsdorf, Switzerland and Harlan Horst Postbus, Netherlands) were induced to ovulate by an intra peritoneal injection of 5

I.U. of PMSG (Pregnant mare serum gonadotropin, Folligon, Intervet), followed 46 h later by an intra peritoneal injection of 5 I.U. HCG (human chorionic gonadotropin: Chorulon, Intervet). Subsequently, donor females were mated with STAT3-MER transgenic male mice. Morulae were flushed from the uterotubal junction 3 days after mating and cultured overnight in M16 medium (Sigma). Fully expanded blastocysts were transferred onto MEF in KSR-KDMEM, KSR-KDMEM without LIF with 1 μM OHT or KSR-KDMEM without LIF and cultured at 37°C in an atmosphere of 10 % CO_2 in air for 6–7 days without media changes. Full-grown ICMs were picked from the outgrown TE and transferred into droplets of trypsin-EDTA solution (Invitrogen) by a mouth-controlled glass-capillary. The ICMs were dissociated mechanically into groups of cells and these aggregates reseeded onto embryonic feeder fibroblasts. 3–4 days later, compact stem cell colonies could be identified. Single colonies were dissociated as described above and reseeded. Subsequently, non-differentiating clonal lines were partly frozen, partly passaged onto 6 cm plates, and split after 2–3 generations for further characterization.

Karyotype and Sex Determination of ES cell lines

For chromosome counts, ES cells were pre-treated for 3 hrs with colcemide (Sigma, 0.05 μg/ml) and metaphase spreads were prepared according to Triman et al. (1975). Sex determination was carried out using primers specific for the smcy (Selected Mouse cDNA on Y) a gene that maps to the short arm of the mouse Y-chromosome and its X-chromosome homolog smcx (Selected Mouse cDNA on X). The primers bind to both homologues but amplify fragments of different sizes on the X and the Y chromosome. Female cell lines exhibit a single band of 341 bp whereas male cell lines exhibit 2 bands of 341 and 312 bp respectively. The following primers were used: SMC4-1: 5'-CTG AAG CCT TTG GCT TTG AGC AAG CTA C-3'; SMCX-1: 5'-CAA AGA ATT TGG CAG CGG TTT CCC T-3'.

Generation of injection chimeras

C57BL/6 host embryos were recovered at the morula stage from the oviducts of hormonally treated females and cultured overnight (see above). Blastocysts were transferred into drops of M16 medium (Sigma) and ES cells into drops of HEPES-buffered ES-medium. About 10–15 ES-cells were injected into each blastocyst. After a recovery period of about 2 hrs, injected blastocysts were transferred into the uterine horns of pseudo pregnant NMRI foster mothers (Harlan, England). Chimeric offspring were identified by the absence of coat color pigmentation. Chimeric males were set up to breed at the age of about 8 weeks.

Western Blotting

ES cell cultures were homogenized with RIPA buffer (50 mM Tris-Cl pH 7.4, 1% NP-40, 0.25% Sodium Deoxycholate, 150 mM NaCl). Protein concentration was determined with BCA-Method (Pierce). Samples were subjected to SDS-PAGE and blotted onto PVDF membranes (Millipore, Volketswil, Switzerland) at 100 V for 1–2 h at 4°C. Immunodetection and chemiluminescent visualization were performed as recommended by the supplier of the chemiluminescence blotting kit (Roche Diagnostics, Rotkreuz, Switzerland). Anti STAT3 antibody (C-20) was purchase from Santa Cruz Biotechnology, anti-phospho (Y705) was purchased from New England Biolabs.

Immunohistochemistry and alkaline phosphatase staining

For alkaline phosphatase staining the cells were washed with CMF-PBS and fixed in 4% paraformaldehyde in PBS. The cells were washed with PBT (PBS with 0.1% triton X) and incubated in alkaline phosphatase buffer (100 mM Tris-Cl pH9.5, 50 mM $MgCl_2$, 100 mM NaCl) containing the AP-substrates nitrotetrazolium-blue and X-phosphate (Roche Diagnostics, Rotkreuz, Switzerland).

For immunohistochemistry the cells were washed in PBS and fixed with 4% paraformaldehyde in PBS. Cells were washed with PBT and incubated with the primary antibodies diluted in PBT containing 2% (v/v) horse serum. Secondary fluorescence labelled antibodies were used for detection. Anti OCT-3/4 (N-19) antibodies were purchased by Santa Cruz Biotechnology, SSEA-1 (Mouse mAb) by Chemicon International and Alexa Fluor 488 anti mouse and Alexa Fluor 594 anti rabbit secondary antibodies were purchased from Molecular Probes.

cRNA labelling and Hybridization of microarrays

cDNA synthesis 1st round of amplification

RNA was combined with 1 μg of T7-dT primer (5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG-(dT)24-3') in a total volume of 12 μl and incubated at 70°C for 10 min. The reaction was placed on ice and the following reagents added in a total volume of 20 μl: first strand buffer (1×), DTT (10 mM), dNTP mix (500 μM), and Superscript III (200 units, Invitrogen) and incubated at 42°C for 2 h. Second strand synthesis was performed by adding the following reagents in a total volume of 150 μl: second strand buffer (1×), dNTP mix (200 μM), *E. coli* DNA ligase (10 units), *E. coli* DNA polymerase (40 units), RNase H (2 units), and incubated at 16°C for 2 h. T4 DNA polymerase (10 units) was then added to fill in the ends of the cDNA and incubated at 16°C for an additional 15 min. Following phenol:chloroform extraction and ethanol precipitation, the cDNA was resuspended in RNase-free water. Transcription was performed using Ambion's (Austin, USA) MEGAscript reagents. A 20 μl

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reaction containing cDNA, NTP mix (7.5 mM), reaction buffer (1×), and 2 μl enzyme mix was incubated at 37°C for 4 h and the primary cRNA was purified using the RNeasy kit (Qiagen, Chatsworth, CA) per the manufacturer's specifications. Two hundred nanograms of the purified cRNA were then carried forward in the secondary amplification. Samples with less than 200 ng cRNA were concentrated to a 5 μl volume and the entire sample used for the next round of amplification.

cDNA synthesis 2nd round of amplification

First-strand cDNA was synthesized by incubating the above cRNA with 1 μg of random primers (Invitrogen, Carlsbad, CA) at 70°C for 10 min. The reaction was then placed on ice and the following reagents added in a 20-μL reaction: first-strand buffer (1×), DTT (10 mM), dNTP mix (500 μM), and Superscript III (200 units). The reaction was incubated at 42°C for 2 h. RNase H (2 units) was added to the reaction and incubated at 37°C for 20 min and then heat-inactivated. The cDNA was combined with 1 μg of the T7-dT primer and incubated at 70°C for 10 min. The reaction was then placed on ice and the following reagents added for a final volume of 150 μl: second-strand buffer (1×), dNTP mix (200 μM), *E. coli* DNA Polymerase (40 units), and then incubated at 16°C for 2 h. T4 DNA polymerase (10 units) was added to fill in the ends of the cDNA and incubated at 16°C for an additional 15 min. The cDNA was purified with phenol:chloroform and precipitated with ethanol.

Biotin secondary IVT

The secondary transcription reaction was performed using the Enzo BioArray HighYield RNA kit (Affymetrix, Santa Clara, CA). The secondary cDNA was incubated at 37°C for 4 h in a 40 μl reaction as indicated by in the manufacturer recommendations and the labelled cRNA purified using the RNeasy kit.

Array Hybridizations and Analysis

The hybridizations were performed with the Murine Genome U74v2 Set, consisting of three GeneChip® probe arrays (U74Av2, U74Bv2 and U74Cv2), together they contain probe sets corresponding to approximately 36,000 full-length mouse genes and EST clusters from the UniGene database. The biotin-labeled cRNA was fragmented in 40 mM Tris-acetate buffer pH 8.1 containing 100 mM potassium acetate and 30 mM Magnesium acetate. 15 μg of every labelled cRNA was mixed with the appropriate buffers and hybridized to respectively a mouse U74Av2, U74Bv2 and U74Cv2 (Affymetrix) for 16 h at 45°C. Computer analysis of the resulting data was performed using the dCHIP software package. Samples were run on triplicate microarrays (three independently cultivated, isolated and labelled WT probes and three independently cultivated, isolated and labelled STAT3-MER probes) and the resulting data combined into subsets and compared using dCHIP. The 18 .CEL files generated by the Affymetrix Microarray Suite (MAS) were converted in .DCP files using dCHIP. The .DCP files were normalized and raw gene expression data generated. Comparison was performed using the dCHIP software wherein the three WT FVB ES cells were designated as "baseline" (B) and the three STAT3-MER overexpressing ES cells designated as "experiment" (E). Genes expressed 1.5 fold higher or lower in the WT versus STA3-MER cells were then identified by defining the appropriate filtering criteria in the dCHIP software (E/B < 1.5 or B/E > 1.5; E-B > 100 or B-E > 100, P < 0.1, t-test). Calculations were performed with both the PM/MM (perfect match/mismatch) difference model and the PM (perfect match) only model in order to compare the results. The microarray data is deposited in the Gene Omnibus Repository (GEO), accession number GSE11398.

Real-Time Quantitative PCR (Q-PCR)

The total RNA from cultured ES cells was obtained using Qiagen RNeasy mini-kit and reverse transcribed with oligo-dT primers (Invitrogen) and SuperscriptIII (Invitrogen). Quantitative Real time experiments were performed with the SyberGreen technology using the QuantiTect SYBR kit (Qiagen) and a Rotorgene 6000 cycler (Corbett). For quantitation of gene expression comparative Ct-method was used after normalization with β-actin. The following primers were used:

βactin_fwd: 5'-cat cca ggc tgt gct gtc cct gta tgc-3'

βactin_bwd: 5'-gat ctt cat ggt gct agg agc cag agc-3'

Lefty2_fwd: 5'-aca gcg cgg atg tgg agg aga tgg-3'

Lefty2_bwd: 5'-atc ctc acg gac tct cag cca ttc a-3'

Eif2s2_fwd: 5'-tac atc gtc aac cca aac atc tcc ttg c-3'

Eif2s2_bwd: 5'-ggc acg gag ctg tgc tcg ctt-3'

Dppa3_fwd: 5'-agg gtc cgc act ttg ttg tcg gtg c-3'

Dppa3_bwd: 5'-gct cct aat tct tcc cga ttt tcg cat-3'

Hexokinase2_fwd: 5'-tgt ggt ggc cgt ggt aaa tg-3'

Hexokinase2_bwd: 5'-tct tga ggc gct ctg aga tg-3'

NDP5211_fwd: 5'-cat gag cag cta cag agg aag ca-3'

NDP5211_bwd: 5'-gtg cct cag att cac tgt gta gct aa-3'

Nanog_fwd: 5'-aca agg gtc tgc tac tga gat gc-3'

Nanog_bwd: 5'-gga gac ttc ttg cat ctg ctg g-3'

Pem/Rhox5_fwd: 5'-ctt ccg tgg aca aga gga ag-3'

Pem/Rhox5_bwd: 5'-tgt cat agc cgg cat atg tg-3'

Ppp1r15b_fwd: 5'-gcc ttc aag ctg gtc tag tc-3'

Ppp1r15b_bwd: 5'-cat cgc tat caa agc cat cg-3'

Pramel6_fwd: 5'-cag gaa gac gag tgg caa agc acg t-3'

Pramel6_bwd: 5'-agc cct gga atc tca tag cct aca tc-3'

Pramel7_fwd: 5'-gag gag aag cag aac atc agc aag a-3'

Pramel7_bwd: 5'-ctc tta gag gcg tga cat cta ggt t-3'

Stat3_fwd: 5'-ggc aag ggc ttc tcc ttc tg-3'

Stat3_bwd: 5'-agc tgc tgc ttg ttg gtg tat gg-3'

Skp2_fwd: 5'-cag ctg ctc cag act gga tg-3'

Skp2_bwd: 5'-ggt tcc ctc tgg cac gat tc-3'

Socs3_fwd: 5'-cct cca gca tct ttg tcg gaa gac-3'

Socs3_bwd: 5'-tac tga tcc agg aac tcc cga atg-3'

Trim41_fwd: 5'-tga gcc gca tgt ttt gtc agg ctg c-3'

Trim41_bwd: 5'-c aca ctt cgc gct gga cta gga gct-3'

Quantitative RT-PCR for each gene was done in triplicates and the values were normalized to the corresponding amounts of β-actin RNAs.

In situ hybridization

Templates for riboprobe-synthesis were obtained by amplification through RT-PCR. Briefly, total RNA was isolated from FVB/STAT3-MER ES cells cultivated in presence of 4OHT and RT was performed with oligo-dT primers. 300–400 bp long fragments containing part of the ORF of the genes of interest were amplified by PCR and cloned in a pCRII® TOPO® dual promoter vector (Invitrogen). DNA templates for riboprobe synthesis were digested with appropriate enzymes to provide fragments for either sense or antisense orientation of the PCR product respect to the vector. Labelled riboprobes were synthesized by SP6 or T7 RNA polymerases by incorporation of digoxygenin-labelled UTPs.

The embryos were transferred in a micro pore insert (12 μm) sitting in a well of a 4 well plate and fixed with freshly prepared 4% PFA/PBS, washed twice in PBT and dehydrated once in 25%, 50%, 75% and twice in 100% methanol/PBT. The dehydration was followed by rehydration in the reverse order of the MeOH/PBT series 75%, 50%, 25% for 5 min each. The embryos permeabilized in RIPA buffer and refixed in 4% PFA/0.2% glutaraldehyde. After prehybridisation in hybridisation solution for ≥2 hours at 70°C the embryos were incubated in hybridisation solution containing 1.6 μg/ml of the corresponding riboprobe. As a control for the specificity of the labelling, in each hybridization experiment control embryos were hybridized with an equal concentration of a sense probe transcribed from the same template as the antisense probe. After high and low stringency washes hybridized riboprobes were detected using an AP-coupled anti-digoxygenin antibodies (Roche Diagnostics, Rotkreuz, Switzerland) and the AP-substrate BM-Purple (Roche Diagnostics, Rotkreuz, Switzerland). The staining reaction was stopped by rinsing in 2 mM EDTA/PBT. Embryos were subsequently post-fixed in 4% PFA/0.1% glutaraldehyde in PBT and cleared in a glycerol:PBT 1:1 solution.

Overexpression of Pramel7, Pem/Rhox5 and Nanog

The full length open reading frames (ORF) of Pramel7 and Pem/Rhox5 were amplified by RT-PCR and sequenced. The full length cDNAs were cloned in the pfloxedNanog expression vector [25] by exchanging the Nanog cDNA with either the one of Pem/Rhox5 or Pramel7. The expression vectors were completely sequenced and finally electroporated in E14 ES cells. After selection with puromycin single colonies were isolated and their expression levels were determined by real time PCR. The clones with the strongest overexpression were chosen for further experiments.

Abbreviations

ES cell: embryonic stem cell; ICM: inner cell mass; LIF: Leukemia Inhibitory factor: MEF: mouse embryonic fibroblasts; OCT-3/4: octamer-binding transcription factor 3/4; OHT: 4-Hydroxytamoxyfen; PRAME: preferentially expressed in melanoma; RTQ-PCR: reverse transcription quantitative PCR; STAT3: signal transducer and activator of transcription 3; STAT3-MER: STAT3 fusioned with a modified estrogen receptor; TE: trophectoderm.

Authors' contributions

PC coordinated the project, conducted the experiments, analyzed the data and drafted the manuscript. EAC, SU and PL participated in performing experiments and acquisition and interpretation of data. TY and TM contributed in preparation of expression vectors. TR generated the transgenic mice by pronuclear injection. BL established the ES cell lines and performed the blastocyst injections. KB participated in coordination of the study and helped to

draft the manuscript. All authors read and approved the final manuscript.

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Annex 2

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Pramel7 Mediates LIF/STAT3-Dependent Self-Renewal in Embryonic Stem Cells

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Key Words. Embryonic stem cells • Differentiation • LIF • STAT3 • Self-renewal

ABSTRACT

A unique and complex signaling network allows ESCs to undergo extended proliferation in vitro, while maintaining their capacity for multilineage differentiation. Genuine ESC identity can only be maintained when both self-renewal and suppression of differentiation are active and balanced. Here, we identify Pramel7 (preferentially expressed antigen in melanoma-like 7) as a novel factor crucial for maintenance of pluripotency and leukemia inhibitory factor (LIF)-mediated self-renewal in ESCs. In vivo, Pramel7 expression was exclusively found in the pluripotent pools of cells, namely, the central part of the morula and the inner cell mass of the blastocyst. Ablation of Pramel7 induced ESC differentiation, whereas its overexpression was sufficient to support long-term self-renewal in the absence of exogenous LIF. Furthermore, Pramel7 overexpression suppressed differentiation in ESCs in vitro and in vivo. This process was reversible, as on transgene excision cells reverted to a LIF-dependent state and regained their capacity to participate in the formation of chimeric mice. Molecularly, LIF directly controls Pramel7 expression, involving both STAT3-dependent transcriptional regulation and PI3K-dependent phosphorylation of glycogen synthase kinase 3β. Pramel7 expression in turn confers constitutive self-renewal and prevents differentiation through inactivation of extracellular signal-regulated kinase phosphorylation. Accordingly, knockdown of Pramel7 promotes ESC differentiation in presence of LIF and even on forced STAT3-activation. Thus, Pramel7 represents a central and essential factor in the signaling network regulating pluripotency and self-renewal in ESCs. STEM CELLS *2011;29:474–485*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

ESCs are derived from the inner cell mass (ICM) of blastocyst [1, 2] and represent an important tool for the study of early embryonic development and the pluripotent state, mostly because of their two distinctive properties, that is, their ability to undergo indefinite mitotic self-renewal and to differentiate into a range of specialized cell types. A tightly balanced interplay between different pathways is necessary to promote self-renewal in ESCs. Some of these pathways act through the repression of factors that initiate differentiation programs [3–7]. STAT3, OCT3/4, SOX2, and Nanog are transcription factors that regulate various aspects of ESC fate and safeguard the maintenance of the pluripotent state [3, 5].

Derivation and maintenance of murine ESCs were originally achieved by using feeders or the cytokine leukemia inhibitory factor (LIF) in combination with fetal calf serum or the growth factor bone morphogenetic protein. LIF acts through the leukemia inhibitory factor receptor/gp130 complex to maintain pluripotency [8, 9]. Cultivation of ESCs on *Lif*-deficient fibroblasts leads to differentiation, indicating that they mostly provide LIF [10]. LIF-independent maintenance of mouse ESCs with retention of pluripotency (adult chimerism) has been previously described for cell lines, which overexpressed Nanog or KLF2 [11, 12]. In vitro, the overexpression of PEM/RHOX5 also maintains pluripotency without LIF, even though contribution to chimera has not yet been proven [13, 14]. Nowadays, it is possible to bypass LIF, feeders, and serum requirement by using two inhibitors (2i conditions) which block mitogen-activated protein kinase (extracellular signal-regulated kinases [ERKs]) and glycogen synthase kinase 3β (GSK3β) [15]. Interestingly, maximal self-renewal is obtained by combination of LIF and 2i confirming LIF/STAT3 signaling as an essential component of self-renewal in ESCs. Despite the importance of this pathway, STAT3 downstream target genes have remained elusive. In a recently performed microarray study, we found Pramel6 and Pramel7 (preferentially expressed antigen in ESCs upon conditional in melanoma like 6 and 7) strongly upregulated in ESCs upon conditional overexpression of STAT3 [14]. These findings indicate a potential role of these genes in the stabilization of ESCs. In this work, we aimed at the functional characterization of Pramel6 and particularly of Pramel7 in ESCs.

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Our results demonstrate that *Pramel7* is a new direct STAT3 target gene, fundamental for the LIF-mediated maintenance of pluripotency and for the inhibition of differentiation.

Materials and Methods

Cell Culture

Medium for E14 129/Ola and transgenic ESCs (complete medium [CM]-medium): Glasgow minimal essential (Sigma, Buchs, Switzerland, www.sigmaaldrich.com/switzerland-schweiz.html), 100 mM sodium pyruvate (Sigma), 10% fetal bovine serum (Gibco, Invitrogen, Basel, Switzerland, www.invitrogen.com), 50 mM β-mercaptoethanol (Gibco), 1× minimal essential medium nonessential amino acids (Gibco), 200 mM L-glutamine. Medium supplemented or not with 1,000 U/ml ESGRO murine LIF (Millipore, Chemikon, Zug, Switzerland, www.millipore.com). N2B27-medium: DMEM/F-12 (Invitrogen), Neurobasal (Invitrogen), 50 mM β-mercaptoethanol (Gibco), 200 mM L-glutamine, N2-Supplement 100× (Invitrogen), B27-Supplement 50× (Invitrogen). Medium supplemented with two inhibitors: 3 μM CHIR99021 (Stemgent, Cambridge, MA, www.stemgent.com) and 1 μM PD0325901 (Stemgent). *Lif* knockout fibroblast: *Lif* +/− mice [16] were mated and at E14.5 the fetuses were isolated. Heads and placentas were used for genotyping, whereas the rest of the embryo was trypsinized and cultured. *Lif* −/− fibroblasts were expanded and treated with mitomycin-C (10 μg/μl). Neural-differentiation medium: DMEM/F-12 (Invitrogen), B27-Supplement 50× (Invitrogen), N2-Supplement 100× (Invitrogen). For PI3K inhibition, 5 μM LY294002 were added to the N2B27 medium not supplemented with CHIR99021 and PD0325901.

Immunohistochemical Analyses

E14, transgenic, and recombined ESCs were cultivated for 11 days with CM-medium with or without LIF on *Lif* knockout feeders. At day 11, all cell lines were analyzed for OCT3/4 and stage-specific embryonic antigen-1 (SSEA-1) expression. For the serum- and feeder-free experiment, the same cells were cultivated on gelatinized plates for 9 days. N2B27 medium was supplemented with either both or one inhibitor or only LIF. ESCs were fixed in 4% paraformaldehyde and incubated overnight at 4°C with primary antibodies against OCT3/4 (rabbit anti OCT3/4, Santa Cruz Biotechnology, Santa Cruz, CA, www.scbt.com) and SSEA-1 (Mouse mAb, Millipore). Secondary fluorescence-labeled antibodies were used for detection (anti rabbit Alexa Fluor 594 and anti mouse Alexa Fluor 488, Molecular Probes, Invitrogen). Nuclei of the cells were counterstained with DAPI (Roche, Basel, Switzerland, www.roche.ch).

Pramel7 Knockdown

shRNA vectors: four specific shRNA vectors against Pramel7 cloned in pGFP-V-RS vector (Origene, Rockville, MD, www.origene.com) and one negative control shRNA pGFP-V-RS vector. Sequences of shRNA are listed in Supporting Information Table 2. ESCs were transiently lipofected with either the shRNA constructs against Pramel7 or with the control vector by using FuGENE HD Transfection Reagent (Roche) and selected with puromycin. Transfection efficiency was monitored by EGFP fluorescence and Pramel7 knockdown was analyzed by real-time polymerase chain reaction (PCR). Both E14 wild-type (wt) and STAT3MER transgenic cells were cultivated on feeders with CM-medium either supplemented with LIF (E14 cells) or with hydroxy-tamoxifen (OHT; STAT3MER cells). STAT3MER cells were analyzed for alkaline phosphatase (AP) expression at day 8, respectively 4 of the knockdown. For STAT3MER cells, all the AP-positive colonies present in the 35-mm dishes were counted. For E14 cells, colonies with greater than 80% staining were classified as "undifferentiated," 20%–80% staining as "mixed," and less than 20% as "differentiated."

LIF Induction Experiment

wt ESCs, established under feeder- and serum-free conditions, were cultivated on gelatinized 35-mm dishes in N2B27 + 2i medium. Cells were then incubated for 4 hours in N2B27 + 2i, N2B27 + PD0325901, N2B27 + CHIR99021, N2B27 + LY294002 or in N2B27 without inhibitors. After 4-hour incubation, LIF was added to the media. Total RNA was extracted after 30 minutes, 1 hour, 3 hours, and 5 hours of LIF incubation. Reverse transcription and real-time PCR were performed.

Part of Material and Methods is provided in the Supporting Information.

Results

Characterization of *Pramel6* and *Pramel7* Genes

In the mouse, *Pramel6* and *Pramel7* cluster on chromosome 2(D) in opposite orientations (Supporting Information Fig. 1A). The amino acid composition of both proteins is very similar (Supporting Information Fig. 1B). Search for recognizable domains in the open reading frame of both genes using SMART (http://smart.embl-heidelberg.de) and myHits (http://myhits.isb-sib.ch/cgi-bin/motif_scan) revealed the presence of conserved leucine-rich repeats (LRRs), which usually participate in protein-protein interactions. The presence of these types of domains and the absence of conserved domains typical for transcriptional factors suggests that the *Pramel* family might not directly regulate gene transcription but rather act via protein-protein interaction.

Pramel6 and Pramel7 Expression Is Restricted to the Late Preimplantation and is Silenced in the Early Postimplantation Embryonic Stages

Whole mount in situ hybridization studies in the preimplantation embryos showed a homogeneous expression of Pramel6 in all cells of the morula and the blastocyst, whereas Pramel7 mRNA distribution was restricted to the interior part of the morula and the ICM of the blastocyst (Supporting Information Fig. 1C). Gene expression analyses of early postimplantation embryos (E6.5) revealed that at this developmental stage *Pramel6* and *Pramel7* genes are silenced (Fig. 1A). At this stage, the embryos expressed, as expected, both Nanog and DPPA3. The Nanog expression was restricted to the embryos, whereas DPPA3 was detected in the embryo as well as in the decidua (Fig. 1A).

In vitro, *Pramel6* and *Pramel7* are constitutively expressed in ESCs (Fig. 1B) independent of whether the cells are cultivated with serum, feeders, and LIF, or with N2B27 + 2i (serum free, feeder free, and LIF free). Expression of both genes disappears as soon as the cells differentiate. In accordance with our previous results [14], the presence of LIF in the medium resulted in an increased expression of both genes, presumably because of a direct activation of STAT3 (Fig. 1B).

Taken together, these observations suggest a possible role of both *Pramel6* and *Pramel7* in the maintenance of pluripotency in vivo as well as in vitro.

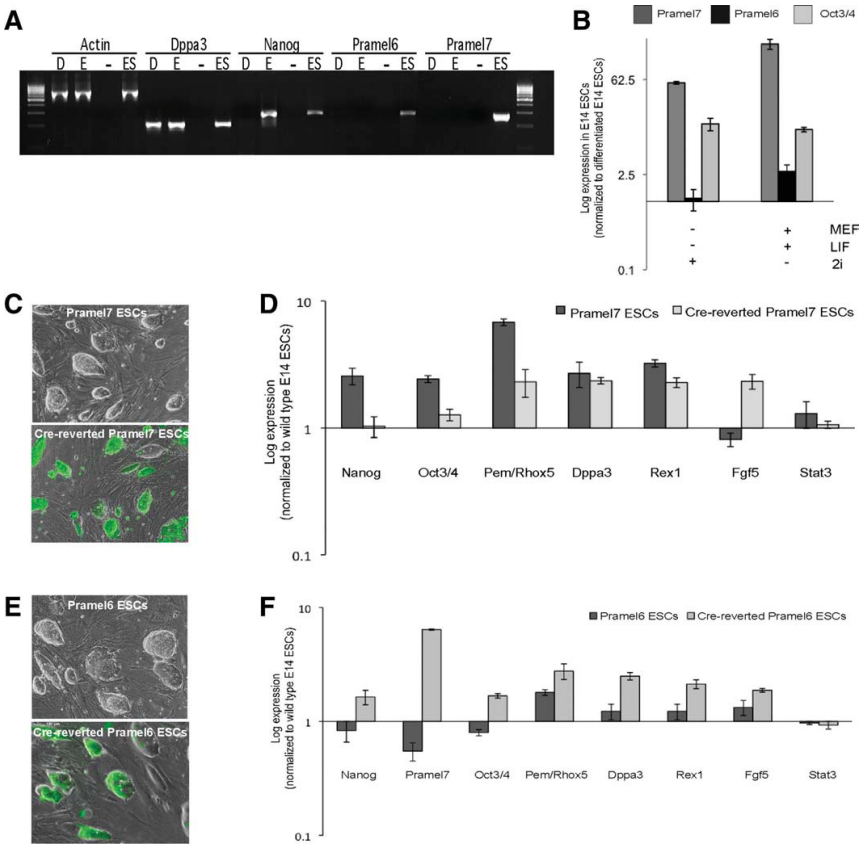


Figure 1. Endogenous expression of Pramel6 and Pramel7 and characterization of the transgenic clones. **(A):** Expression of Pramel6, Pramel7, Dppa3, and Nanog in early postimplantation embryos (E6.5). Pramel6 and Pramel7 expressions are absent in both decidual and embryonic tissue but present in ESCs. Negative control (−). **(B):** Real-time polymerase chain reaction (PCR) analysis of wild-type (wt) ESCs cultivated in presence of LIF and feeders (MEF) or in presence of the extracellular signal-regulated kinases and glycogen synthase kinase 3 inhibitors (2i). Expression levels of Pramel7, Pramel6, and Oct3/4 are compared with differentiated ESCs. **(C, E):** Representative morphology of Pramel7 and Pramel6 overexpressing cells and Cre-reverted (EGFP expressing) ESCs cultivated in presence of LIF and feeders. **(D, F):** Real-time PCR analyses of known pluripotency-related genes in Pramel7, Pramel6, and in Cre-reverted ESCs. Data were normalized to the expression level in the wt ESCs. Abbreviations: D, decidual tissue; E, embryonic tissue; ES, ESC; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblasts.

Pramel7 but Not Pramel6 Overexpression in ESCs Induces General Upregulation of Known Pluripotency-Related Genes

We generated ESCs conditionally overexpressing LoxP-flanked open reading frames of *Pramel6* or *Pramel7*, which can be excised by Cre-recombinase simultaneously bringing *egfp* under the CAG promoter (Supporting Information Fig. 2A). Overexpression experiments were performed in E14 129/Ola (E14) ESCs and, if not specified, they were cultivated in presence of feeders and serum (referred as CM). All clones showed a high expression of the transgene, which reverted to wt levels once recombined (Supporting Information Fig. 2B and 2C). All transgenic clones showed the classical morphology of pluripotent ESCs (Fig. 1C, 1E, and Supporting Information Fig. 2E) and expressed the pluripotency markers AP, SSEA-1, and OCT3/4 (data not shown). On the transcriptional level, as assessed by real-time PCR, the overexpression of Pramel7 induced a slight increase of most of the pluripotency genes (Fig. 1D). Accordingly, on Cre-recombination the expression of Nanog and OCT3/4 reverted to wt levels in combination with an increase in fibroblast growth factor 5 (FGF5) (Fig. 1D). Overexpression of Pramel6 did not induce significant changes on the expression of the investigated genes (Fig. 1F). Nanog overexpression did not influence the transcriptional levels of Pramel7, indicating that *Pramel7* is not a target of Nanog (Supporting Information Fig. 2D).

Overexpression of Pramel7 Liberates ESCs from LIF Dependence and Maintains Pluripotency

We further analyzed the ability of ESCs overexpressing either Pramel6 or Pramel7 to be propagated clonally on *Lif*-knockout feeders [16] without LIF in the medium. After 11 days, E14 ESCs exhibited widespread differentiation and no longer expressed the pluripotency markers OCT3/4, SSEA-1, and AP (Fig. 2A, 2B and Supporting Information Fig. 3A). In contrast, Pramel7-overexpressing cells (referred as Pramel7 cells) were undistinguishable from Nanog-overexpressing cells (Supporting Information Fig. 3A) and only less than 10% of

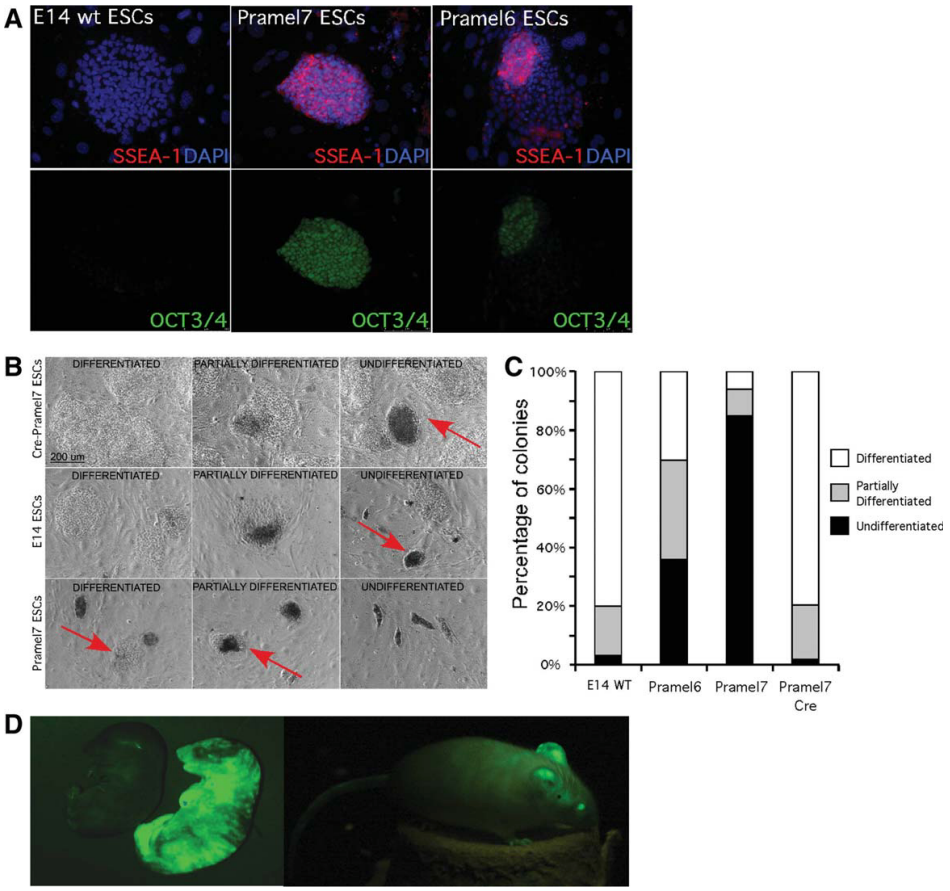


Figure 2. Characterization of Pramel7 and Pramel6 transgenic ESCs. **(A):** Immunostaining for OCT3/4 and SSEA-1 in E14 wt, Pramel6 and Pramel7 overexpressing ESCs cultivated for 11 days in absence of leukemia inhibitory factor (LIF) on a layer of *Lif* knockout feeders. Magnification: ×20. **(B, C):** Alkaline phosphatase (AP) staining and quantification of AP-positive colonies after 11 days in absence of LIF (100 colonies were counted). **(D):** Contribution of Pramel7 Cre-deleted cells to embryo and adult chimeric animals. Chimerism was assessed by green fluorescence. Wild-type pup (EGFP negative), EGFP-positive Cre-reverted Pramel7 pup and the adult chimera are shown. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; SSEA-1, stage-specific embryonic antigen-1.

the colonies showed no AP activity (Fig. 2C). Accordingly, Cre-recombined Pramel7 ESCs (referred as Cre-Pramel7 cells) behaved like wt ESCs and exhibited more than 80% of differentiated colonies (Fig. 2C). In contrast, only about one-third of the colonies in Pramel6-overexpressing clones remained completely undifferentiated (Fig. 2C and Supporting Information Fig. 3A). Our data reveals that overexpression of Pramel7, but not of Pramel6, is sufficient to liberate ESCs from LIF dependence and to promote self-renewal.

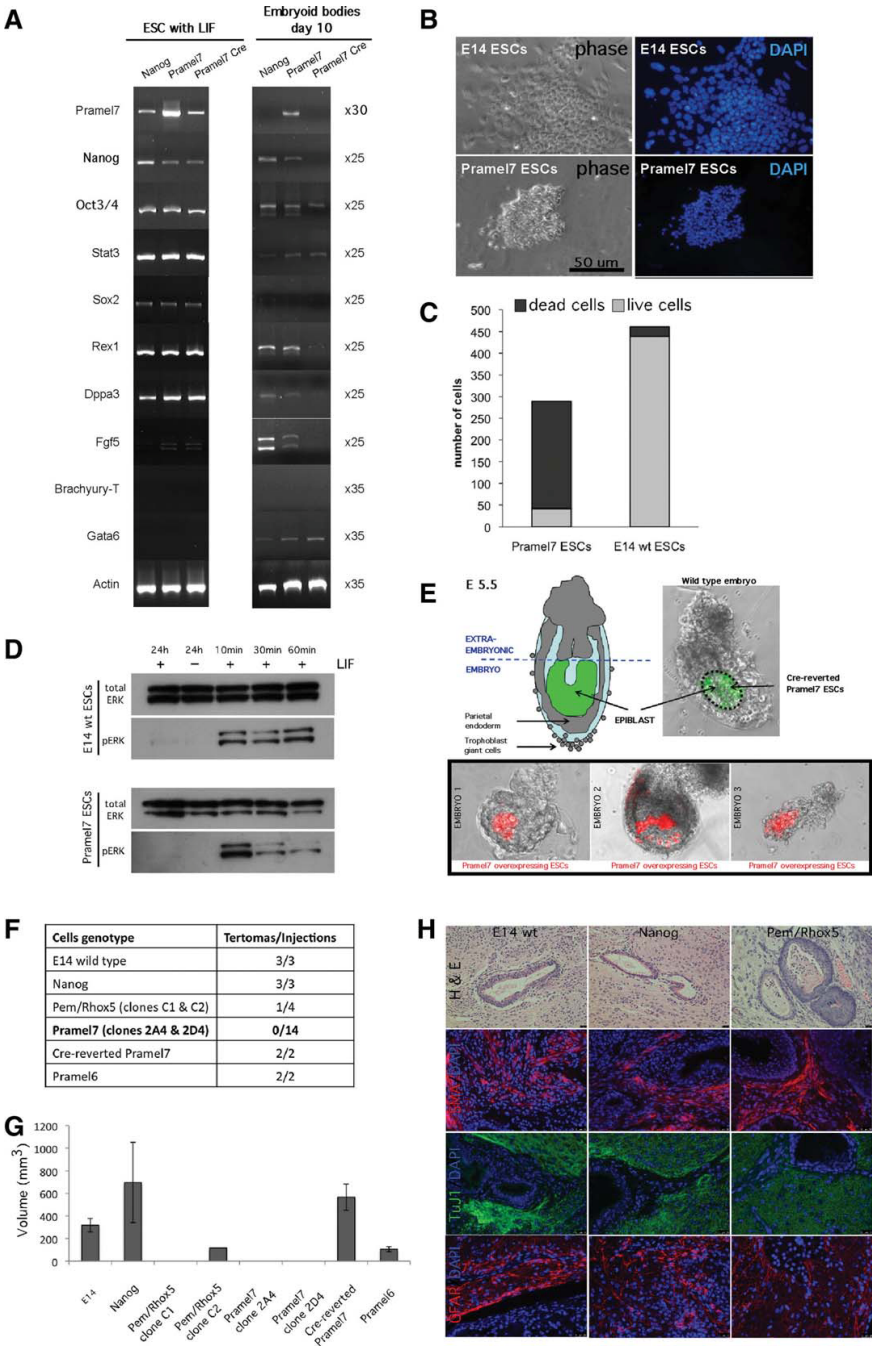
Finally, we investigated whether cells maintained in a self-renewing pluripotent state exclusively through overexpression of Pramel7 were retaining their embryo colonization capacity. Excision of *Pramel7* restored LIF dependence and these cells contributed to the generation of healthy live chimeras upon morula aggregation (Fig. 2D and Supporting Information Table 3).

Pramel7-Overexpressing ESCs Fail to Differentiate In Vitro

To investigate the role of Pramel7 in ESC differentiation, embryoid bodies were generated with Pramel7, Cre-Pramel7, and Nanog-overexpressing ESCs. As assessed by reverse transcription and PCR, expression of pluripotency markers, such as OCT3/4, Nanog, and REX1, was persisting after 10 days in Pramel7 cells as well as in the Nanog-overexpressing cells, whereas it was completely abolished in Cre-revertants (Fig. 3A). To further assess the capacity of Pramel7 cells to undergo defined differentiation, cells were cultivated with neural differentiation medium. E14 cells differentiated, whereas the majority of Pramel7 cells showed a large number of cells with pyknotic nuclei (Fig. 3B, 3C) indicating a high degree of cell death.

Pramel7 Maintains Pluripotency In Vitro Through Gradual Suppression of ERK Phosphorylation

ERK phosphorylation is known to be an early signaling event required for the differentiation of ESCs, and it has been proposed that the balance between LIF-induced activation of STAT3 and ERK may determine the efficiency of self-renewal and thereby influence stem cell fate [17, 18]. To further understand the mechanism underlying Pramel7-mediated



Cells genotype	Tertomas/Injections
E14 wild type	3/3
Nanog	3/3
Pem/Rhox5 (clones C1 & C2)	1/4
Pramel7 (clones 2A4 & 2D4)	**0/14**
Cre-reverted Pramel7	2/2
Pramel6	2/2

Figure 3. Pramel7 overexpression results in differentiation defects in vitro and in vivo. **(A):** Expression levels of pluripotency genes (Pramel7, Nanog, Oct3/4, Pem/Rhox5, Stat3, Sox2, Rex1, Dppa3), endoderm (Gata-6), primitive ectoderm (Fgf5), and mesoderm (Brachyury-T) were measured by semiquantitative RT-PCR in ESCs (control) and in embryoid bodies generated from Pramel7-overexpressing cells, Pramel7-Cre revertants, and Nanog-overexpressing cells. Number of PCR cycles is annotated. **(B, C):** In vitro neural differentiation of Pramel7-overexpressing cells and parental E14 cells. Pramel7-overexpressing clones exhibited an extensive number of cells with pyknotic nuclei, as shown by DAPI staining. Magnification: ×20. Number of live and dead cells after 4 days of neural differentiation: Cells which showed condensed nuclei were defined as dead cells, whereas normal nuclei were considered as live cells. **(D):** Western blotting for phospho-Erk after LIF stimulation of wt and Pramel7-overexpressing ESCs for 10, 30, or 60 minutes reveals that Pramel7 maintains pluripotency through gradual suppression of pERK. **(E):** Morula aggregation of Cre-reverted Pramel7 cells (EGFP positive) and Pramel7-overexpressing cells (RFP labeled). Pramel7-overexpressing cells do not correctly take part to the development, whereas Cre-reverted Pramel7 cells integrate into the developing embryo and at E5.5 they are part of the epiblast. **(F–H):** Teratoma formation reveals impaired in vivo differentiation potential of Pramel7-overexpressing cells. Efficiency of teratoma formation in E14 wt ESCs, Nanog, Pem/Rhox5, Pramel6, and Pramel7-overexpressing cells was assessed by number of teratomas generated **(F)** and tumor volume (mm^3) measurement **(G)**, as well as by degree of tumor differentiation **(H)** analyzed by immunostaining for SMA, TuJ1, and GFAP. Magnification: ×10. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acid protein; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblasts; pERK, phosphorylated ERK; SMA, smooth muscle actin; TuJ1, βIII-tubulin.

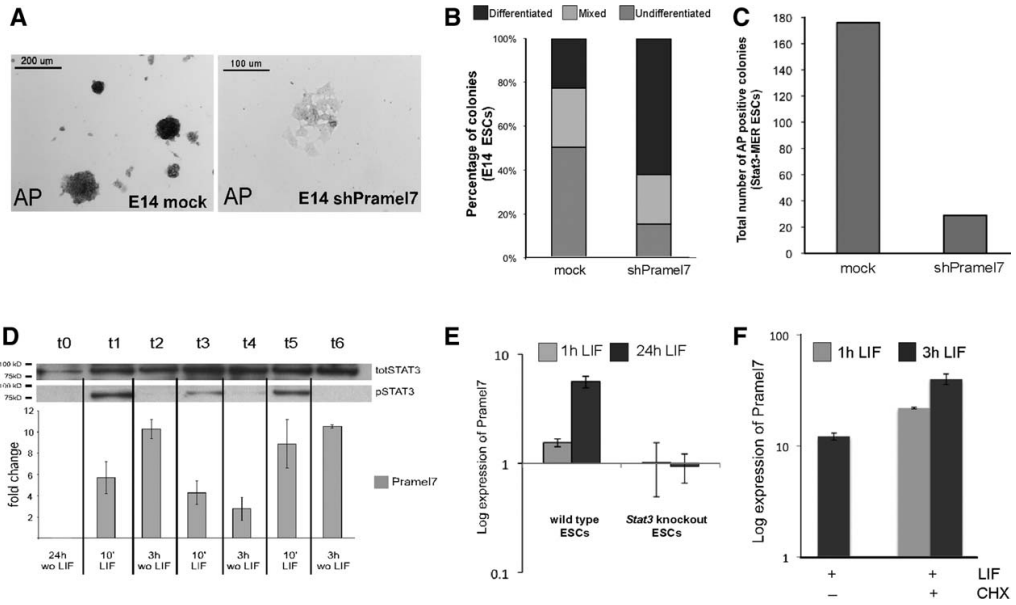


Figure 4. Pramel7 expression is indispensable for LIF/STAT3-dependent maintenance of pluripotency and is a direct STAT3 target gene. **(A, B):** Representative appearance of AP-stained colonies **(A)** after Pramel7 knockdown in E14 ESCs in presence of LIF. Control (E14 mock), Pramel7 knockdown (E14 shPramel7). Quantification of AP-positive colonies **(B)** after Pramel7 knockdown in E14 wt cells. Colonies that showed more than 80% positive cells were identified as "undifferentiated," 20%–80% as "mixed," and less than 20% as "differentiated". **(C):** Pramel7 knockdown in STAT3MER cells cultivated in presence of 4-hydroxy-tamoxifen. Total number of AP-positive colonies is presented. **(D):** Time-pulse assay with LIF in E14 wt ESCs. Phosphorylation levels of STAT3 (pSTAT3) were monitored by Western blot, whereas the amount of Pramel7 mRNA was measured by real time-polymerase chain reaction (PCR). **(E):** Real-time PCR analysis of Pramel7 mRNA expression in wt and *Stat3* null ESCs cells after LIF stimulation for 1 or 24 hours. **(F):** STAT3 directly transcribes *Pramel7* gene. Real-time PCR analysis of Pramel7 mRNA expression in E14 wt ESCs deprived from LIF for 24 hours followed by LIF stimulation for 1 or 3 hours in the presence or absence of cycloheximide (50 μg/ml). Abbreviations: AP, alkaline phosphatase; LIF, leukemia inhibitory factor.

self-renewal, we investigated by Western blotting activation of ERK in E14, Pramel6, Pramel7, and in Cre-recombined ESCs on LIF stimulation. Twenty-four hours LIF-depleted wt, Pramel6-, and Cre-Pramel7 cells showed progressive phosphorylation of ERK (pERK) on LIF induction (Supporting Information Fig. 3B). In Pramel7 cells, however, pERK was only detectable 10 minutes after LIF stimulation and drastically decreased after 30 minutes (Fig. 3D). Taken together, this data suggests that Pramel7 overexpression prevents ESCs from differentiation by promoting ERK dephosphorylation.

Pramel7 Cells Are Unable to Form Teratomas and to Contribute to Embryo Development

To test the capacity of Pramel7 cells to contribute to embryo development, we genetically labeled these cells with red fluorescent protein (RFP) and performed morula aggregations. After being transferred into the uterus of foster mothers, at E5.5 the embryos were isolated and analyzed. Embryos aggregated with Pramel7-RFP cells exhibited malformations (Fig. 3E and Supporting Information Table 3), indicating that Pramel7 cells do not enter normal development.

We further investigated the ability of Pramel6 and Pramel7 cells to form teratomas. We transplanted 10^6 ESCs subcutaneously into immunoincompetent nonobese diabetic/severe combined immunodeficiency mice (Fig. 3F). After 3 weeks, wt and Cre-Pramel7 ESCs produced teratomas of similar size (Fig. 3G and Supporting Information Fig. 4A) containing derivatives of all three germ layers (Supporting Information Fig. 4B). Pramel6 cells generated similar teratomas but with smaller volume than the ones generated by the control clones (Fig. 3G and Supporting Information Fig. 4A, 4B). Intriguingly, 14 independent injections with two different Pramel7 ESC-clones overexpressing similar amounts of Pramel7 never generated teratomas, even after 2 months incubation. Seeing that no previous publication describes a similar behavior of pluripotent cells, we tested the potential of teratoma formation of ESCs overexpressing Nanog or PEM/RHOX5. A total of 100% of the injections with Nanog-overexpressing ESCs produced teratomas undistinguishable in size (Fig. 3G) and histological composition from the ones generated from wt and Cre-revertant cells (Fig. 3H, Supporting Information Fig. 4A and 4B). This data indicates that Nanog overexpression is not sufficient to prevent differentiation in the context of teratoma.

ESCs-overexpressing PEM/RHOX5 were previously shown to form teratomas containing undifferentiated cells [13]. Interestingly, 75% of the injections with PEM/RHOX5-overexpressing cells failed to generate teratomas, in a similar way to the Pramel7 cells (Fig. 3G). Nevertheless, the only teratoma isolated contained differentiated tissue similar to the wt and Nanog cells (Fig. 3H). In summary, overexpression of Pramel7 impairs the capacity of ESCs to generate teratomas and the inability to differentiate probably causes the death of the cells.

Pramel7 Is Necessary for LIF- and STAT3-Dependent Maintenance of Pluripotency in ESCs

To investigate whether Pramel7 is required for maintenance of pluripotency in ESCs, we performed knockdown experiments in E14 wt ESCs cultivated in the presence of LIF.

Knockdown was achieved by transient ESCs transfection with a cocktail of four shRNA-containing vectors specific for Pramel7. Four days after transfection, Pramel7 mRNA was completely silenced, although the efficiency decreased 4–5 times in comparison with the control cells at day 6 (data not shown). Despite the presence of LIF, knockdown of Pramel7 induced loss of AP activity and differentiation (Fig. 4A, 4B), whereas control cells did not. The data indicates that LIF-mediated self-renewal in ESCs depends on Pramel7 expression.

To further assess whether self-renewal induced by STAT3 overexpression also depends on Pramel7, we exploited STAT3MER ESCs, which conditionally express a STAT3 fusion protein with a mutated estrogen receptor. These cells maintain pluripotency in the absence of LIF by the sole activation of STAT3MER with 4-OHT [19] and were shown to upregulate Pramel7 [14]. Surprisingly, STAT3MER activation failed to promote stem cell maintenance in Pramel7 knockdown cells, resulting in a drastic decrease in the total number of AP-expressing colonies (Fig. 4C). These results clearly indicate that the expression of Pramel7 is essential for both LIF- and STAT3-mediated maintenance of pluripotency.

Pramel7 Is a Novel Direct Downstream Target of STAT3 in the LIF/STAT3 Pathway

On the basis of our previous [14] and recent results, we assessed whether Pramel7 might represent a novel effector of the LIF/STAT3-pathway. We therefore first monitored STAT3 phosphorylation and Pramel7 mRNA expression in response to three cycles of 10 minutes of LIF incubation. Depletion of LIF for 24 hours (t0) and successive incubation with LIF-containing medium for 10 minutes (t1) induced in E14 cells a fast phosphorylation of STAT3 (pSTAT3) as assessed by Western blotting (Fig. 4D). Concomitantly, mRNA levels of Pramel7 strongly increased during this period (Fig. 4D). The same behavior was observed also after the third round of LIF incubation, suggesting that *Pramel7* is a downstream target of LIF/STAT3.

To test the robustness of this hypothesis, we monitored Pramel7 expression after LIF induction in *Stat3* null cells [12] cultivated in N2B27 + 2i medium without LIF. In the wt cells, LIF induced an upregulation of Pramel7 mRNA, whereas *Stat3* knockout cells failed to regulate Pramel7 expression (Fig. 4E). These results clearly indicate that *Pramel7* transcription is STAT3-dependent. Furthermore, LIF stimulation in wt ESCs, in the presence of the protein synthesis inhibitor cycloheximide, resulted in strong activation of *Pramel7* transcription (Fig. 4F) confirming that STAT3 directly drives *Pramel7* transcription.

Pramel7 Expression Is Regulated Through the Parallel Circuitry of the LIF Signal Pathways

We observed that within 10 minutes LIF induces a rapid upregulation of Pramel7 transcripts under standard culture conditions (Fig. 4D). Interestingly, the presence of 2i causes a significant delay in the LIF-dependent Pramel7 upregulation (Fig. 4E). In the presence of 2i, LIF supply is dispensable and the cells can be propagated in the absence of active LIF/STAT3 pathway [15]. We therefore considered whether the retarded responsiveness of *Pramel7* transcription to LIF was due to the absence of active STAT3 or to the presence of the ERK- and/or GSK3β-inhibitors.

We examined induction of Pramel7 on addition of LIF in absence of 2i or in presence of either PD0325901 (ERK-inhibitor) or CHIR99021 (GSK3β-inhibitor). ESCs were then incubated for 30 minutes, 1 hour, 3 hours, or 5 hours with LIF. Intriguingly, the presence of CHIR99021 completely blocked *Pramel7* transcription (Fig. 5A) indicating that the inhibition of GSK3β activity impairs STAT3-mediated transcription of *Pramel7*. We therefore analyzed the effect of LY294002, a phosphoinositide-3-kinase (PI3K) inhibitor on *Pramel7* regulation. Because of the known apoptotic effect of PI3K inhibition, cells were incubated with low inhibitor concentrations and for a short time. STAT3 mRNA expression was not influenced by the presence of the PI3K inhibitor and as expected, increased during the LIF incubation times. Whereas the level of Pramel7 transcripts remained unaltered even after 5 hours of LIF stimulation (Fig. 5B). We can therefore conclude that *Pramel7* transcription is mediated by the parallel activity of the LIF/STAT3 and the PI3K/GSK3β pathways.

It is known that the sole inhibition of ERK or GSK3β is not sufficient for maintaining ESCs undifferentiated. To test whether Pramel7 overexpression can overcome this situation, we cultivated wt and Pramel7 cells in N2B27 medium supplemented only with CHIR99021. After 5 days, wt cells showed significant upregulation of the differentiation markers Brachyury-T, GATA6, and GATA4, and after 9 days, the expression of REX1, Nanog, OCT3/4, and SOX2 was reduced (Fig. 5C). Immunohistochemistry for OCT3/4 and SSEA-1 showed a reduced expression in wt cells and colonies started to spread out loosing their compact shape (Fig. 5D). In contrast, Pramel7 cells formed high compact colonies, which were homogeneously positive for both pluripotency markers (Fig. 5D). Moreover, they formed more than 70% undifferentiated, homogenously AP-positive colonies, whereas E14 cells were only about 25% (Fig. 5E). This demonstrates that overexpression of Pramel7 in combination with the GSK3β-inhibitor is sufficient to maintain ESCs undifferentiated, indicating that the presence of Pramel7 can compensate for the need of MEK/ERK inhibition.

Pramel7 Overexpression Under Feeder- and Serum-Free Conditions Is Only Partially Able to Maintain Pluripotency

We asked next if Pramel7 is able to maintain ESCs undifferentiated in N2B27 medium in the absence of feeders. E14 and Pramel7 ESCs were adapted to the N2B27 + 2i conditions (without LIF) by extensive passaging and both cell lines after 11 passages showed homogenous AP staining (Fig. 6A). We therefore used these cells for our further experiments. We first investigated if overexpression of Pramel7 is sufficient for prolonged cell expansion in N2B27 medium without 2i and LIF, a condition that normally does not support self-renewal. After two passages at clonal density, wt cells died, whereas Pramel7 cells could be split for one more passage, but finally they also died (Fig. 6B). This indicates that the sole overexpression of Pramel7 is not sufficient for prolonged cells expansion under these conditions. We therefore investigated if Pramel7 cells were able to self-renew in N2B27 medium supplemented with LIF alone, a condition that is also normally not sufficient for maintaining ESCs. After passaging, almost all the E14 cells either died or stopped to self-renew. However, Pramel7 cells showed higher self-renewing rate and formed round, compact colonies (Fig. 6C). OCT3/4 and SSEA-1 expression was detected only in the transgenic cells, but not in the wt E14 cells (Fig. 6D). Quantification for AP-positive colonies showed that Pramel7 cells exhibited 5% of completely undifferentiated colonies and about 50% of partially differentiated colonies when stained for AP (Fig. 6E). In contrast, more than 80% of the wt cells were completely differentiated and showed no AP activity at all (Fig. 6E). This was also

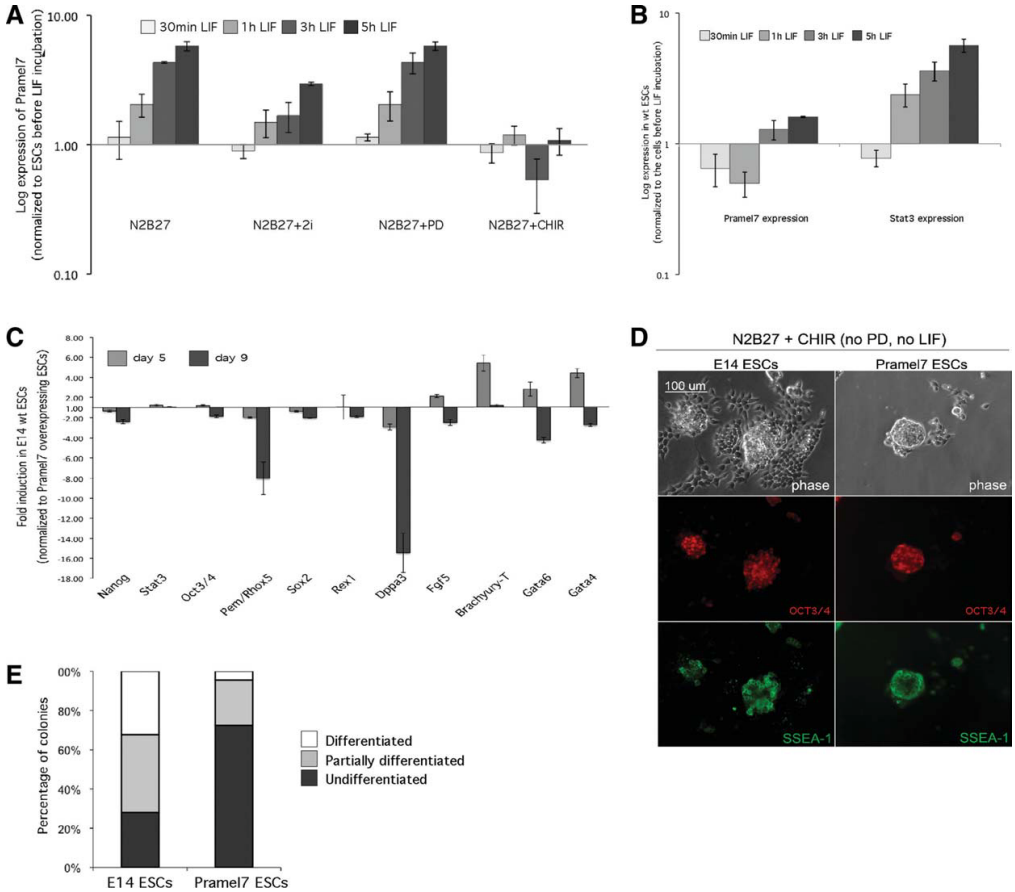


Figure 5. Transcriptional regulation of Pramel7. **(A):** Quantitative real-time polymerase chain reaction (PCR) analysis of Pramel7 gene expression in E14 wild-type (wt) ESCs stimulated with LIF in presence of 2i, PD0325901 (PD), or CHIR99021 (CHIR) at different time points. **(B):** Quantitative real-time PCR analysis of Pramel7 and Stat3 expression in E14 wt ESCs stimulated with LIF in presence of LY294002. **(C):** Gene expression analyses in E14 wt cells cultivated for 5 or 9 days in the presence of CHIR99021. **(D):** Immunostaining for SSEA-1 and OCT3/4 of E14 and Pramel7 cells after 5 days in N2B27 medium supplemented only with CHIR99021. Transgenic Pramel7 cells retained the expression of OCT3/4 and SSEA-1 markers, whereas E14 wt cells differentiated. **(E):** Quantification of alkaline phosphatase-positive colonies after 9 days of culture in N2B27 medium supplemented only with CHIR99021. Abbreviation: LIF, leukemia inhibitory factor.

confirmed by real-time PCR (Fig. 6F). In summary, the combination of Pramel7 overexpression and LIF increases self-renewal capacity of ESCs, facilitating the maintenance of the undifferentiated state. Nevertheless, this synergistic effect was only partial, as complete elimination of differentiation was not observed.

Discussion

The JAK/STAT3-pathway was shown to be essential and sufficient in mouse ESCs to mediate LIF signals thereby contributing to the maintenance of pluripotency [19, 20]. Even though a complete bypass of LIF signaling is possible under certain circumstances [15], optimal self-renewal is obtained by combination of LIF and 2i, confirming LIF/STAT3-pathway as an essential component in ESCs. Moreover, STAT3-activation was recently described to be a limiting factor for reprogramming to ground state pluripotency [21]. Expression of Pramel6 and Pramel7 was increased upon overexpression of STAT3 in ESCs [14] and therefore represent potential candidates involved in regulating the homeostasis of ESCs [14, 22, 23].

Here, we demonstrate that *Pramel7* is not only a direct target of STAT3 but also that STAT3-mediated maintenance of pluripotency strongly depends on Pramel7 expression. During the preimplantation embryo stages the LIF/gp130 pathway is dispensable for early development without diapause [24]. Nevertheless, even in the absence of LIF stimuli, Pramel7 is expressed in the inner part of the morula and in the ICM of the embryo. Intriguingly, in *Stat3*-knockout cells, where the LIF/STAT3-pathway is not active, Pramel7 expression was upregulated compared with the wt cells. These findings suggest that there might be LIF/STAT3 compensatory mechanisms or factors, which drive and regulate Pramel7 expression. We exclude the possibility of an autoregulatory activity of Pramel7, as the protein lacks the characteristic domains

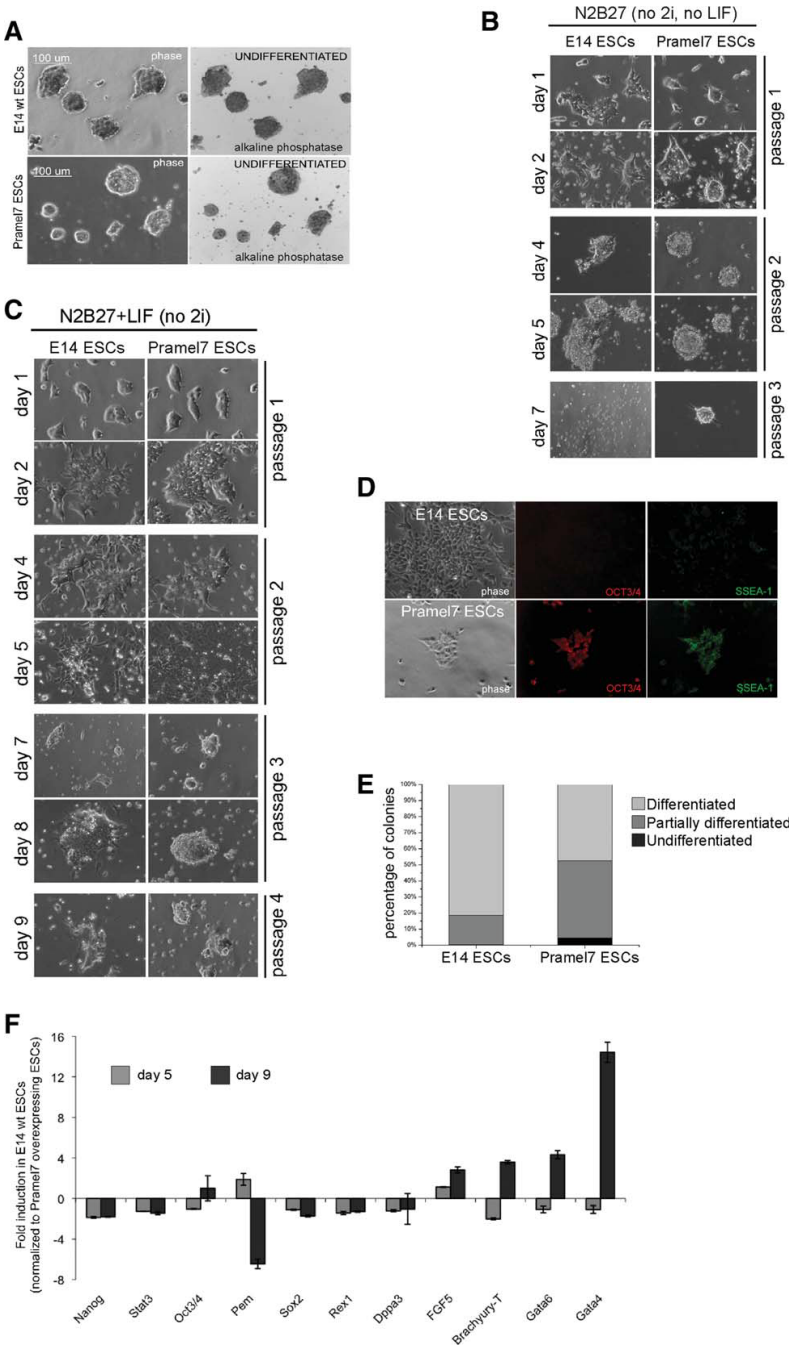


Figure 6. The sole Pramel7 overexpression in serum- and feeder-free condition is not completely sufficient for maintaining the undifferentiated state of ESCs. **(A):** Representative appearance of alkaline phosphatase (AP)-positive colonies in E14 wt and Pramel7-overexpressing cells after 11 passages in N2B27 + 2i medium (feeders free and LIF free). Both cell lines show AP activity. **(B):** Representative morphology of wt and Pramel7 ESCs after three passages in N2B27 medium in absence of 2i and LIF. **(C):** E14 and Pramel7 cells cultivated for four passages in N2B27 medium supplemented with LIF only. **(D):** Immunostaining for OCT3/4 and SSEA-1 expression in wt and Pramel7-overexpressing cells cultivated in N2B27+LIF. E14 cells lost OCT3/4 and SSEA-1 expression, whereas Pramel7 cells remained undifferentiated. **(E):** Quantification of AP-positive colonies after 9 days of culture in N2B27 medium supplemented only with LIF. **(F):** Gene expression analysis of ESCs cultivated 5 and 9 days in N2B27+LIF. E14 cells showed progressively downregulation of pluripotency genes and upregulation of differentiation marker genes. Abbreviations: LIF, leukemia inhibitory factor; SSEA-1, stage-specific embryonic antigen-1.

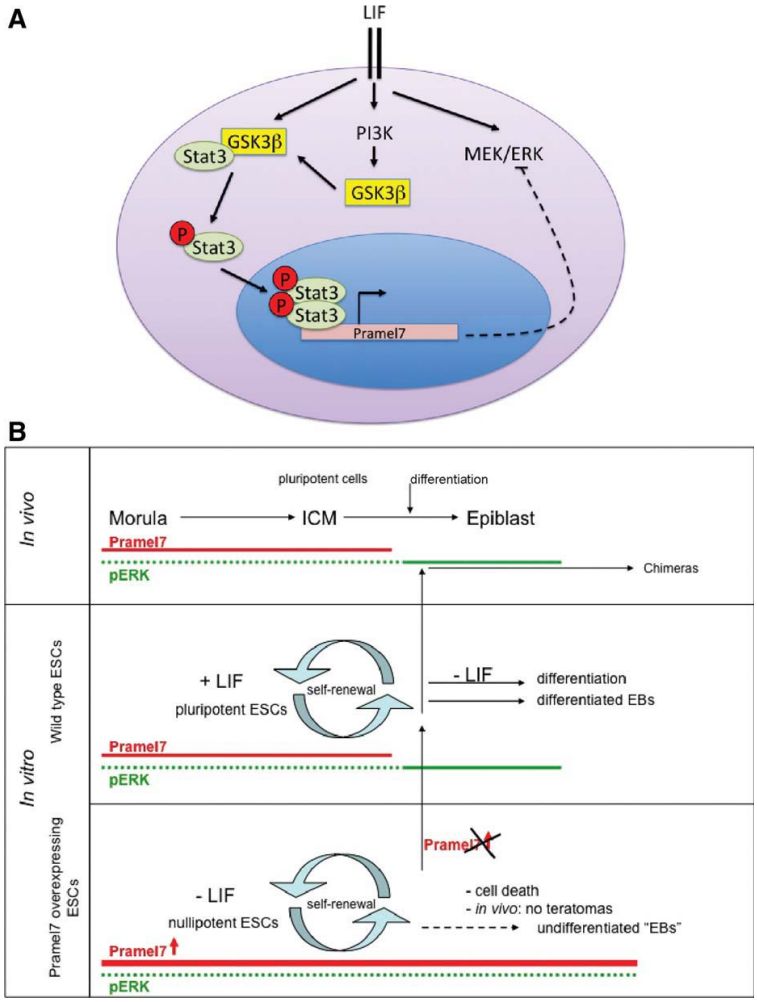


Figure 7. Hypothetical transcriptional mechanisms, which drive Pramel7 expression. **(A):** Schematic representation of *Pramel7* transcription directly controlled by the LIF/STAT3-pathway and also by the LIF/PI3K-pathway. LIF/gp130 receptor leads to the activation of three different pathways, that is, the LIF/STAT3, the LIF/PI3K/GSKβ3, and the LIF/MEK/ERK-pathway. *Pramel7* transcription is directly controlled by the transcription factor STAT3, whereas its phosphorylation is probably regulated by the GSK3β kinase. The combinatorial effect of LIF/STAT3 and GSK3β drives and controls *Pramel7* transcription, which in turn blocks phosphorylation of ERK and therefore ESCs differentiation. **(B):** Schematic representation of Pramel7 expression and effects in vivo and in vitro. In the late preimplantation embryo, Pramel7 expression is only detectable in the inner part of the morula and in the ICM of the blastocyst. Immediately after implantation Pramel7 expression disappears, when differentiation processes occur in the embryo. In vitro wild-type (wt) ESCs do express Pramel7 but need LIF for self-renewing. After LIF depletion wt ESCs differentiate into different cell types and also form EBs. In Pramel7-overexpressing cells, addition of LIF in the medium is not necessary anymore for maintaining the undifferentiated and self-renewing state of the cells. These cells are not able to differentiate and either form undifferentiated embryoid bodies or die. Once the overexpression of Pramel7 is reverted to the wt level, these cells are able to differentiate and take part to embryo development. Abbreviations: EBs, embryoid bodies; ERK, extracellular signal-regulated kinase; ICM, inner cell mass; LIF, leukemia inhibitory factor; MEK, MAPK/ERK Kinase; pERK, phosphorylated ERK.

typical for transcriptional factors but contains LRR domains mediating protein-protein interactions [25].

Pramel7 regulation occurs through a parallel circuit involving both the STAT3 and the PI3K-pathway. The regulation of GSK3β by PI3K is involved in the transcription of *Pramel7* upon LIF stimulation and thus for the maintenance of LIF-mediated control of ESC self-renewal. We suggest that GSK3β is directly involved in the control of STAT3-mediated *Pramel7*-transcription, but the exact nature of this regulation remains to be elucidated. Interestingly, recent data suggests that STAT3-activation is dependent on GSK3β [26]. Beurel et al. reported that GSK3β does not function upstream of STAT3-activating tyrosine kinases but instead is required for the recruitment of STAT3 to the receptor and for its tyrosine phosphorylation-mediated activation. Even though the authors did not directly analyze ESCs, they demonstrated that the dependence of STAT3-activation on GSK3β was a widespread regulatory interaction observed with multiple stimuli and in several types

of cells [26], suggesting that the same molecular process likely occurs in ESCs. This is interesting because it was previously shown that in ESCs, STAT3 is not a target of PI3K action and that the loss of self-renewal and the consequent differentiation of the cells after inhibition of PI3K was due to an increase in pERK upon LIF-stimulation [27]. The ERK-pathway is continuously activated in undifferentiated ESCs predominantly by signaling through FGF receptor [28]. It is widely accepted that in self-renewing ESC cultures, the provision of LIF and the following activation of STAT3 acts downstream of pERK to override the auto inductive capacity of FGF4. In this study, we identify for the first time Pramel7 as a protein that links the three LIF/gp130-induced pathways (LIF/STAT3, LIF/MEK/ERK, and LIF/PI3K/GSK3β). We suggest that the concerted activity of STAT3 and GSK3β controls *Pramel7* transcription, which in turn regulates the phosphorylation of ERK leading to an abrogation of ESC differentiation (Fig. 7A).

Importantly, Pramel7 blocks teratoma formation capacity and differentiation potential in vitro and in vivo, indicating that *Pramel7* silencing is an absolute requirement for differentiation (Fig. 7B). Similar to Pramel7 overexpression, forced expression of Nanog confers LIF-independent self-renewal and prevents differentiation of ESCs [11]. Unlike Pramel7-overexpressing cells, though, Nanog-overexpressing clones were able to generate teratomas-containing derivatives of all three germ layers. Even though forced expression of both genes in absence of LIF leads to self-renewal of ESCs, our data highlights a different reaction to differentiation stimuli and therefore a different function of these genes in maintaining pluripotency. Nanog is expressed at similar levels in both ICM and ESCs, whereas Pramel7 expression is higher in the ICM than in ESCs [22]. Moreover, it was reported that the essential function of Nanog is to define the pluripotent pool of cells of the ICM and the germ cells, and once the cells are established, its function is dispensable, so that *Nanog* null ESCs are able to self-renew [29]. Our data also confirms the idea that Nanog is important for the pluripotent identity of the ICM and of ESCs, whereas Pramel7 is more probably involved in allowing/blocking the start of differentiation rather than actively taking part in the processes maintaining pluripotency. In agreement with this hypothesis, elimination of Pramel7 expression by knockdown induces ESC differentiation independently if LIF or even STAT3 overexpression is present. Taken together, this suggests that Nanog is priming a cell to become pluripotent, whereas Pramel7 inhibits a pluripotent ESC from commitment and suggests that Pramel7 acts as the judge in the trial between pluripotency and differentiation. Its presence maintains the cells in a self-renewing state by retarding commitment.

Assuming that Pramel7 is not directly acting as a transcriptional factor but solely through binding to other proteins, further experiments aimed at the identification of potential Pramel7 binding partners are necessary to reveal the mechanisms underlying maintenance of pluripotency through Pramel7.

Conclusion

Our data show that the combined activity of STAT3 and GSK3 controls *Pramel7* transcription, which in turn regulates the phosphorylation of ERK leading to the inhibition of ESC differentiation. Accordingly, Pramel7 ablation causes ESC differentiation, whereas its overexpression sustains long-term self-renewal in the absence of LIF. These observations prove Pramel7 as an essential factor of the signaling network regulating pluripotency and self-renewal in ESCs.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Supplementary Data

Materials and methods

Endogenous Pramel7 expression in postimplantation embryos

Embryos were isolated at E6.5 from time-mated females. Six embryos were pooled together and total RNA was extracted from the decidua and from the embryos (QIAGEN RNeasy mini-kit). 0.3 µg of total RNA were reverse transcribed and cDNAs were used for RT-PCR. Primers are listed in Supplemental Table 1.

Generation and characterization of transgenic ESCs

Vector cloning, generation and characterization of transgenic ESCs overexpressing the selected genes were performed as previously described (Cinelli et al., 2008). Primers for real-time PCR are listed in Supplemental Table 1. The Pramel6 clones P6-2 and P6-9 and the Pramel7 clones P7-B5 and P7-2D4 possessed the strongest overexpression and were chosen for further experiments. For Cre-excision, overexpressing ESCs were transiently electroporated and further examined by fluorescent microscopy for EGFP expression. Single colonies were picked and analyzed by real-time PCR. The clones with wt-like gene expression were chosen for *in vitro* experiments and for generation of chimeras.

Long-term culture under serum- and feeder-free conditions

E14 and Pramel7 cells were cultivated on gelatinized dishes with N2B27+2i medium and passaged till the feeders were eliminated. After 11 passages both cell lines were tested for AP activity. Cells were then cultivated with N2B27-medium supplemented either with 2i, only CHIR99021 or only LIF for 9 days. At day 5 and 9 cells were taken for RNA extraction and for immunohistochemical analyses. Total RNA was reverse transcribed and gene expression was monitored by real-time PCR.

Real-time PCR

0.5 to 2 µg of total RNA from cultured ESCs were reverse transcribed with Oligo-dT primers (Invitrogen) and Superscript III (Invitrogen). Real-time PCR was performed in triplicates and analyzed with Delta Ct-method. β-Actin was used for normalization. Primers are listed in Supplemental Table 1.

Western blotting

Cells were homogenized with RIPA buffer (50 mM Tris-Cl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl). Protein concentration was determined with BCA-Method (Pierce). Samples were subjected to SDS-PAGE and blotted onto PVDF membrane (Millipore, Volketswil, Switzerland) at 100 V for 1.5h at 4°C. Immunodetection and chemiluminescent visualization were performed as recommended by the supplier of the chemiluminescence blotting kit (Roche Diagnostics, Rotkreuz, Switzerland). Each lane was loaded with 10µg of protein. Antibodies: against STAT3 (C-20, Santa Cruz Biotechnology), against phosphorylated STAT3 (Y705, New England Biolabs), anti ERK and phosphorylated ERK (Thr202/Tyr204, Cell Signaling Technology).

***In vitro* differentiation**

Embryoid bodies were maintained in suspension for 10 days in CM-medium without LIF on bacteriological Petri dishes. At day 10 total RNA was isolated, reverse transcribed and RT-PCR was performed. Primers are listed in Supplemental Table 1.

For monoculture neural differentiation, $2x10^5$ ESCs were plated onto gelatinized 35mm dishes. The cells were cultivated for 4 days with neural differentiation medium. At day 4 cells were fixed and stained with DAPI. For pyknotic nuclei quantification: 10 pictures of the cells were randomly taken with 40x magnification. Every single cell was counted as dead when the nucleus was condensed, as live if the DAPI staining showed normal morphology of the nuclei.

Teratoma formation

$1x10^6$ cells were injected subcutaneously into each dorsal flank of NOD/SCID mice. Three weeks after injection, teratomas were dissected and fixed in 4% paraformaldehyde. Sections were stained with haematoxylin/eosin, anti bIII-Tubulin (Sigma), anti smooth muscle actin (Sigma), and with anti

glial fibrillary acid protein antibodies (Sigma). The Veterinary Office of the Canton of Zurich, Switzerland approved all animal experiments.

Chimera generation with Cre-reverted Pramel7 cells

Cre-recombined EGFP expressing ESCs were aggregated with CD-1 morula according to standard procedures. Embryos were transferred into the uterine horns of pseudo pregnant CD-1 females. Chimerism of the pups was assessed by visualizing EGFP fluorescence. Number of the obtained chimeric animals is listed in Supplemental Table 3. The Veterinary Office of the Canton of Zurich, Switzerland approved all animal experiments.

TABLE 1: primer used

Gene name	Sequence
βactin_bwd	5'-gat ctt cat ggt gct agg agc cag agc-3'
βactin_fwd	5'-cat cca ggc tgt gct gtc cct gta tgc-3'
Brachyury_bwd	5'-aga ggc tgt aga aca tga tt-3'
Brachyury_fwd	5'-atg cca aag aaa gaa acg ac-3'
Dppa3_bwd	5'-gct cct aat tct tcc cga ttt tcg cat-3'
Dppa3_fwd	5'-agg gtc cgc act ttg ttg tcg gtg c-3'
Fgf5_bwd	5'-ctt cag tct gta ctt cac tgg-3'
Fgf5_fwd	5'-aaa gtc aat ggc tcc cac gaa-3'
Gata6_bwd	5'-ctc ttg gta gca cca gct ca-3'
Gata6_fwd	5'-gca atg cat gcg gtc tct ac-3'
Nanog_bwd	5'-gga gac ttc ttg cat ctg ctg g-3'
Nanog_fwd	5'-aca agg gtc tgc tac tga gat gc-3'
Oct3/4_bwd	5’-ctc gaa cca cat cct tct ct -3’
Oct3/4_fwd	5’-ggc gtt cgc ttt gga aag gtg ttc -3’
Pem/Rhox5_bwd	5'-tgt cat agc cgg cat atg tg-3'
Pem/Rhox5_fwd	5'-ctt ccg tgg aca aga gga ag-3'
Pramel6_bwd	5'-agc cct gga atc tca tag cct aca tc-3'
Pramel6_fwd	5'-cag gaa gac gag tgg caa agc acg t-3'
Pramel7_bwd	5'-ctc tta gag gcg tga cat cta ggt t-3'
Pramel7_fwd	5'-gag gag aag cag aac atc agc aag a-3'
Rex1_bwd	5'-agg gaa ctc gct tcc aga ac-3'
Rex1_fwd	5'-aga aag cag gat cgc ctc ac-3'
Sox2_bwd	5'-ttg cct taa aca aga cca cga aa-3'
Sox2_fwd	5'-tag agc tag act ccg ggc gat ga-3'
Stat3_bwd	5'-agc tgc tgc ttg ttg gtg tat gg-3'
Stat3_fwd	5'-ggc aag ggc ttc tcc ttc tg-3'

TABLE 2: shRNA against Pramel7 sequences

shRNA	**Sequence**
GI580382	GGA GCA GTA GAA GTG TCT AAG GTC AGG AA
GI580383	AGC CTG GTG AGG AAT GAA GTA TTG ACC GT
GI580384	AAT GTA TCC TGC TCC ATC AGA GGT CTA TA
GI580385	TCA CTC AAG AGG AGA AGC AGA ACA TCA GC

TABLE 3: List of Morula Aggregations

Morula aggregation with Cre-reverted Pramel7 ESCs

	transferred embryos	obtained chimeras
Aggregation for isolation of E17.5 embryos	102	6
Aggregation for postnatal chimera formation	40	3

Morula aggregation with RFP-labeled Pramel7 overexpressing cells

ESCs used for aggregation	Aggregate embryos transferred to foster mother	Chimeric embryos (E5.5) **normal**	Chimeric embryos (E5.5) **abnormal**
Cre-reverted Pramel7 cells (EGFP)	65	8	-
Pramel7 overexpressing ESCs (RFP)	103	-	10

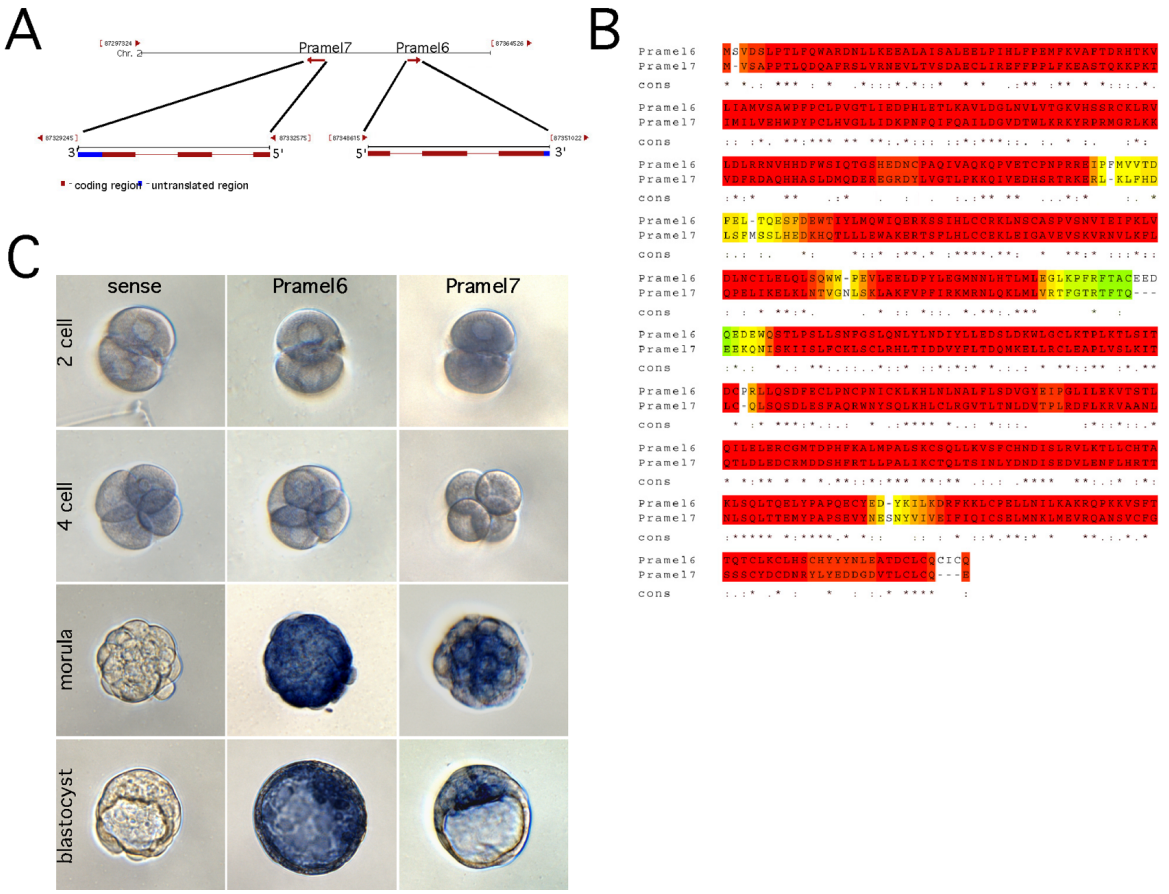
Supplemental Figure 1: *In silico* characterization of *Pramel6* and *Pramel7* genes and expression in the preimplantation embryo. **A**. Localization of *Pramel6* and *Pramel7* genes on chromosome 2(D) in the mouse. The genes are oriented in opposite directions and separated from a genomic fragment of 16 kb. **B**. Aminoacid alignment of Pramel6 and Pramel7 proteins shows high similarity (51%) and the presence of highly conserved leucine-rich repeats (LRRs). The alignment was generated with TCoffee and shaded with respect to Pramel6, red to yellow to green indicates the degree of conservation of the aminoacids, being red the highest and green the lowest degree. **C.** *In situ* hybridization of Pramel6 and Pramel7. Pramel6 exhibits a general expression in all cells of the morula and of the blastocyst whereas Pramel7 expression is restricted to the cells of the inner part of the morula and to the ICM in the blastocyst. Sense probes in the same concentration of the antisense probes were used as negative controls for the hybridization. Magnification 40x.

Supplemental Figure 2: Generation and characterization of transgenic ESC lines. **A**. Scheme of floxed *Pramel*'s expression vector: The LoxP sites are positioned in the second exon of the CAG cassette and between the terminator sequence and *egfp*, such that following Cre-mediated recombination CAG directly controls expression of *egfp*. **B**. Real-time PCR analysis of Pramel7 overexpressing clones and their respective Cre-revertants. Fold changes were measured compared to the wt E14 cells. Actin was used for normalization. **C**. Pramel6 overexpressing clone with the respective Cre-revertant. Pramel6 expression was assessed by real-time PCR. **D**. Gene expression in Nanog overexpressing clone. Real-time PCR analyses of known pluripotency related genes. **E**. Representative morphology of wt E14 and the transgenic clones (Cre-reverted Pramel7 clone 2D4_3; Pramel7 overexpressing cells clone 2D4; Pramel6 overexpressing cells clone 2; Nanog overexpressing cells).

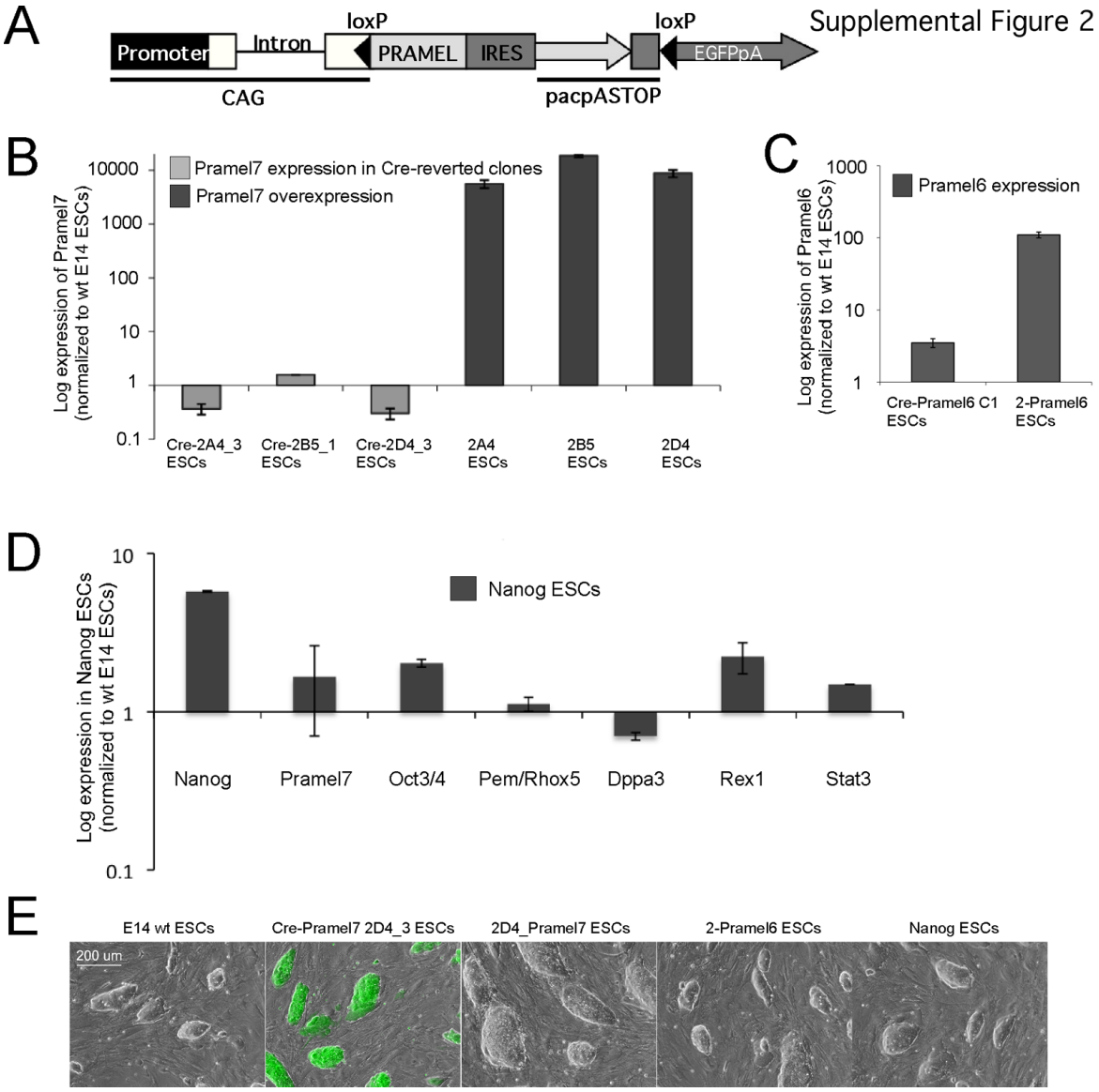
Supplemental Figure 3: A. Phase pictures of all the transgenic ESC lines. Parental E14 cells, Cre-reverted Pramel7, Pramel6, Pramel7 and Nanog overexpressing cells were cultivated on *Lif* knockout feeders in absence of LIF in the medium for 11 days. **B.** Western blot for LIF-dependent phospho-Erk levels. Pramel7 maintains pluripotency through gradual suppression of ERK phosphorylation. E14 wt cells, Pramel6, and Cre-reverted Pramel7 cells showed gradual phosphorylation of ERK increasing with the LIF incubation times. Only Pramel7 overexpressing cells showed dephosphorylation of ERK after 30min incubation with LIF. Total ERK was used as a loading control.

Supplemental Figure 4: A. Size of teratomas generated with Nanog-overexpressing cells (Nanog), E14 wt cells, Cre-reverted Pramel7 cells (P7Cre) and Pramel6 overexpressing cells (P6). **B**. Various tissues present in teratomas derived from E14 wt cells, Cre-reverted Pramel7 cells, Nanog overexpressing and Pramel6 overexpressing cells. Magnification 10x.

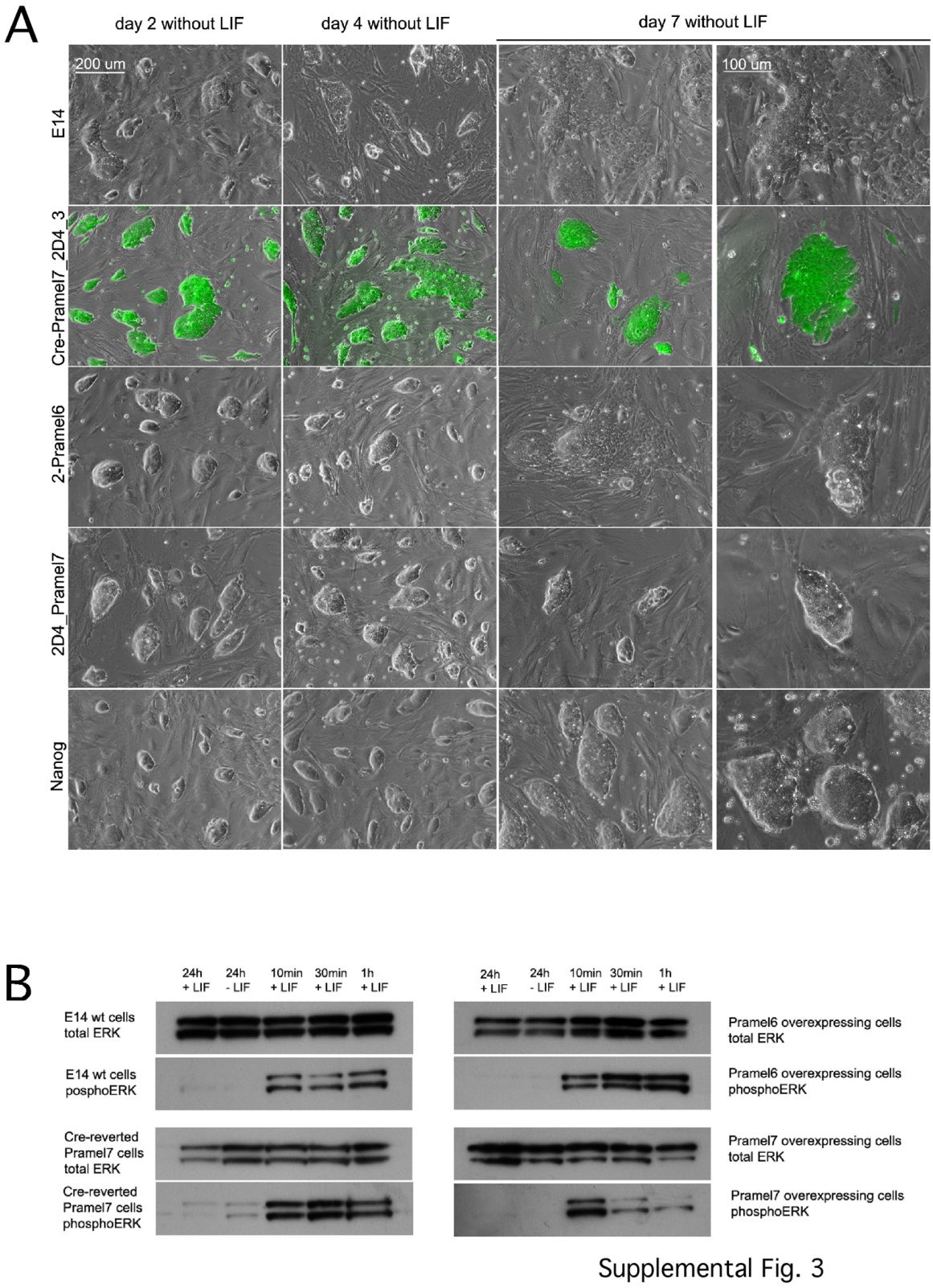
Supplemental Figure 1



Supplemental Figure 2

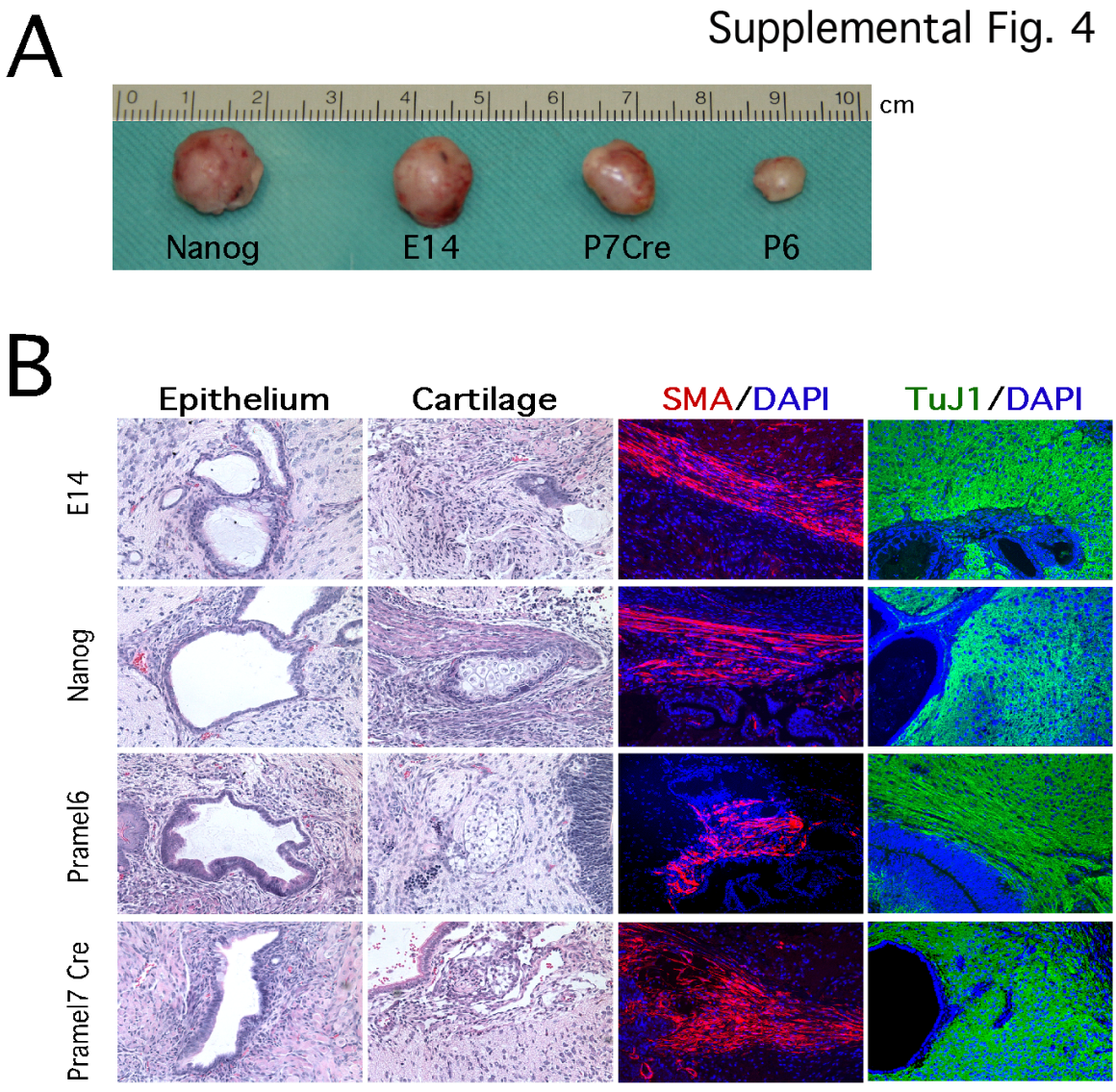


Supplemental Figure 3



Supplemental Fig. 3

Supplemental Figure 4



Annex 3

Cross-species genome wide expression analysis of late mouse and rat preimplantation embryos

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Abstract

The transition between morula and blastocyst stage during preimplantation development represents the first differentiation event of embryogenesis. Morula cells undergo the first cellular specialization and produce two well-defined populations of cells, the trophoblast and the inner cell mass (ICM). Embryonic stem cells (ESCs) with unlimited self-renewal capacity are believed to represent the *in vitro* counterpart of the ICM. Both mouse and rat ESCs can be derived from the ICM cells, but their *in vitro* stability differs.

In this study we performed a microarray analysis in which we compared the transcriptome of mouse and rat morula, blastocyst, and ICM. This is the first study investigating the gene expression changes during the transition from morula to blastocyst in the rat preimplantation development. Moreover, it represents a new example of statistical approach for cross species analysis, applicable also to other species comparisons, that allows to highlight the species-specific behavior of genes within important pathways and families. In order to identify alternative regulation of important molecular mechanisms the investigation of differential gene expression between the two species was extended at the level of signaling pathways, gene families, and single selected genes of interest. This study reports for the first time that in the pluripotent pool of cells of the rat and mouse preimplantation embryo substantial differential regulation of genes is present, which might explain the difficulties observed for the derivation and culture of rat ESCs using murine conditions. Some of the genes differentially expressed are already known to be important factors in the maintenance of pluripotency in ESCs, like for example *Sox2* or *Stat3*, or play a role in reprogramming somatic cells to pluripotency like *c-Myc*, *Klf4* and *p53* and would therefore represent interesting candidates to further analyze *in vitro* in the rat ESCs.

Author Summary

In this study we analyzed the molecular mechanisms controlling the transition between morula and blastocyst during preimplantation development in mice and rats in order to identify differences in the establishment of the pluripotent pool of cells in both species. Comparing the molecular processes taking place during the transition from morula to blastocyst in the mouse and in the rat represents a good model for understanding the differences in derivation and cultivation of ESCs observed in the two species.

In the present work cross-species comparison allowed the identification of differences in the regulation of important developmental pathways and factors, and of genes involved in the maintenance of pluripotency and reprogramming. These results are of interest for the optimization of the derivation and cultivation of rat ESCs.

Introduction

The period of time that lasts from the fertilization of the egg to the implantation of the blastocyst represents an attractive model for studying regulatory networks that determine cell fate decisions. Of particular interest is the transition between morula and blastocyst stages, the first differentiation event of embryogenesis, a period where pluripotent cells are formed. Morula cells undergo the first cellular specialization and produce an outer rim of cells, the so-called trophoblast that surrounds an inner core of cells the inner cell mass (ICM). The signals that regulate the differentiation of the trophectoderm are largely unknown. One of the key discoveries of the last century was the observation that after transferring blastocyst stage embryos in an artificial context it is possible to establish cells, which retain the pluripotent state. These cells, also known as embryonic stem cells (ESCs) are derived from the ICM of the blastocysts (Evans and Kaufman, 1981; Martin, 1981) and exhibit unique characteristics: They unlimitedly self-renew *in vitro* and are able to contribute to the formation of all cells of an adult organism. Understanding how this population of cells is formed and maintained is of fundamental importance not only for developmental biology but also for regenerative medicine and cancer biology. Nowadays, ESCs are routinely derived from mouse blastocyst embryos, even though

not with any difficulties. The mouse has represented for many years the sole organism where pluripotent and germline competent ESCs could be derived. Only recently, almost 30 years after the establishment of the first murine ESC line, genuine rat ESCs have been generated (Buehr et al., 2008; Li et al., 2008). The real identity and stability of these cells is not yet completely understood, especially because murine ESCs and rat ESCs are derived and cultivated under different conditions. Mouse ESCs can be maintained in medium containing inhibitors of the fibroblast growth factor (FGF)/mitogen-activated protein kinase (MEK)/extracellular signal-related kinase (ERK1/2) and of the glycogen synthase kinase 3 (GSK3) inhibitor. These culture conditions are known as the 3i or 2i culture conditions (Ying et al., 2008) and have been also used for the successful establishment of murine ESCs from non-permissive mouse strains such as the non-obese diabetic (NOD) mice (Nichols et al., 2009a). Molecularly, rat ESCs express the same pluripotency markers like murine ESCs (Buehr et al., 2008; Li et al., 2008) but can be established and maintained *in vitro* only under defined culture conditions and additionally in the presence of LIF and feeders. The difference between the two species is also mirrored at the preimplantation development level. Mouse embryos reach the blastocyst stage at day E3.5 whereas the rat at day E4.5 (Fig. 1A), nevertheless both species give birth at day E21. These differences highlight the complexity of the mechanisms that define the pluripotent state of a cell and let to assume that in the rat other molecular mechanisms might be involved in the maintenance of the pluripotent state *in vitro* compared to the mouse.
Extending the knowledge of the molecular processes driving the establishment of pluripotency *in vivo* is decisive for understanding the identity and properties of ESCs *in vitro*. We therefore reasoned that a comparison of the gene expression profiles in preimplantation embryos in the mouse and in the rat would be of advantage for improving the comprehension of the pluripotent state and eventually for optimizing derivation and cultivation of rat ESCs. With this purpose we examined and compared with a molecular genetic approach the global gene expression in morula, blastocyst, and in isolated ICM of mouse and rat. With this cross species gene expression comparison we were able to highlight different regulation not only of important developmental pathways like Wnt and Notch, but also of genes known to play important roles in the maintenance of pluripotency in ESCs and in reprogramming processes like for example *Sox2*, *Klf4*, *c-Myc* and *p53*.

Results and Discussion

Statement of grounds and experimental design

During early embryogenesis, pluripotency is a characteristic property of a distinct number of cells of the morula and the ICM of the blastocyst, from where pluripotent ESCs are established (Fig. 1B). We collected morula and blastocysts stage embryos from mouse and rat and, by immunosurgery, we isolated the ICM cells from the blastocysts. All the embryos and ICMs were pooled into two groups for every developmental stage (Fig. 1C). Pooling of embryos for RNA extraction in this study was chosen mainly because of the low amounts of RNA that can be isolated from preimplantation embryos, and in addition because of the heterogeneity of the cell populations present in the embryos. For the analysis we pooled a large number (n>100) of the independent isolated embryos to achieve a sufficient accuracy of biological pooling (Fig. 1C). Due to the difficulties to isolate a larger number of embryos from mice and rats, we performed the microarray study by using two replicate samples per developmental stage (Fig. 1D). Data obtained in such a way are not sufficient to compute p-values from statistical tests, but allows quantification based on fold changes of gene expression between the three different cell populations. The direction, magnitude and shape of the profile of fold changes were used as a main basis for the whole genome inter-species comparison (Fig. 1D).
The global significance analysis of the mouse and rat expression profiles is depicted on the Figure 2A and 2B. Top 20 differentially expressed probesets for the mouse (Fig. 2A) and for the rat (Fig. 2B) have been selected in each of the pairs of treatments and then used at the input of hierchical clustering for the heatmap. The heatmap shows that each pairwise comparison has a group of upregulated and downregulated genes, however on the global level there is hardly any overlap in terms of orthologs (just one gene in common in the heatmap built with over 50 genes from the three top 20 lists). This leads to the conclusion that prior biological knowledge should be used for the search of meaningful relationships. We therefore gathered the information present in the GeneGo pathways in order to investigate the similarities and differences locally, within the context of pathways and gene families.

Identification of differentially expressed genes in the three cell populations

To reveal similarities and differences in the regulatory mechanisms controlling mouse and the rat development of morula and blastocyst, we first analyzed the data of the mouse and the rat microarray study separately. We selected the genes that had a fold change (understood as the difference between log2 signal values) higher than 1.5 in the three comparisons: ICM versus blastocyst (ICM vs B), blastocyst versus morula (B vs M), and ICM versus morula (ICM vs M). The genes with a positive value are always upregulated in the first cell population of the comparison, whereas those with a negative value are higher expressed in the second cell population of the comparison.
For the mouse study we found that out of 17'973 genes 166 were differentially regulated between the ICM and the blastocysts (Fig. 2C and Supplemental Table 1A). A higher number of differentially regulated genes was found in the comparison B vs M, where 1'621 genes had a fold change higher than 1.5 (Fig. 2C and Supplemental Table 1B). In the comparison ICM vs M we found 1'370 genes, 957 of which were also differentially expressed between blastocyst and morula (Fig. 2C and Supplemental Table 1C). Between the 957 genes found in both the comparisons ICM vs M and B vs M a clear upregulation of the transcription factor *Stat3* (Signal Transducers and Activators of Transcription 3) and the Lif receptor *Lifr* were present. Both genes were upregulated in the blastocyst or the in ICM compared to the morula but were not differentially expressed in the comparison ICM vs B (Supplemental Table 1B and 1C), confirming their specific expression in the blastocyst. This is interesting because previous data suggested that the LIF/STAT3 pathway is dispensable during the preimplantation embryo development in the absence of diapause (Li et al., 1995; Nakashima et al., 1999; Nichols et al., 2001; Stewart et al., 1992). Nevertheless, this pathway plays a fundamental role *in vitro* in the maintenance of pluripotency and derivation of ESCs (Casanova et al., 2011b; Cinelli et al., 2008; Matsuda et al., 1999; Niwa et al., 1998). Our data highlight the possibility that the characteristic expression of these genes at the blastocyst stage might indeed play an important role, and that the previously performed studies with knockout embryos could have been biased by the induction of compensatory mechanisms.
Only 23 genes were differentially expressed in both the comparisons ICM vs B and B vs M (Fig. 2C). Between them, the *Celf5* gene, a member of the CELF gene family (Ladd et al., 2001), showed a 3.5 fold upregulation in the comparison B vs M and a -2.5 fold downregulation in ICM vs M, indicating a potential function in the trophoblast cells of the blastocyst.
In order to reduce the number of differentially expressed genes we further selected for mouse genes with a fold change higher than 3 (Fig. 2D) aiming at the identification of genes that are characteristically expressed in the ICM. The gene *Fos* had a fold change of 3.5 in the comparison ICM vs B and -3.9 in the comparison B vs M (Supplemental Table 1A and 1B) indicating that *Fos* expression is high in the morula and persists in the ICM cells of the blastocyst. Interestingly, it has been shown that *Fos* is also expressed *in vitro* in undifferentiated ESCs and disappears as soon as the cells undergo differentiation (Yang et al., 2009). A second gene with characteristic ICM expression is *Egr1*, which was upregulated 4 times in the comparison ICM vs B and was strongly downregulated in the comparison B vs M (Supplemental Table 1C). This data suggests that *Egr1* plays an important role in the mouse ICM.

For the rat study we identified 192 out of 12'102 genes that had a fold change higher than 1.5 in the comparison ICM vs B (Supplemental Table 2A), among these 71 were also found differentially regulated in the comparison ICM vs M (Fig. 2E) and 7 of them showed a fold change higher than 3 (Fig. 2F). The genes *Nqo1*, *Ddhd1*, *Hmox1* and *Chac1* had a positive fold change in both the comparisons ICM vs B and ICM vs M (Supplemental Table 2C), indicating that they are upregulated exclusively in the ICM cells of the rat blastocyst. None of these 7 genes was found in the mouse study in the comparison ICM vs B and ICM vs M, except for the *Nqo1* (NAD(P)H quinone oxidoreductase) that was upregulated in the ICM compared to the morula, nevertheless with a factor of 1.5. It has been shown that inhibition of NQO1 causes degradation of p53 in various cell types (Asher et al., 2001) therefore NQO1 supports the accumulation of p53, which leads to the induction of growth arrest (Levine, 1997; Vogelstein et al., 2000) and/or apoptosis (Lowe et al., 1993). The finding that in the rat ICM *Nqo1* is upregulated compared to the blastocyst with a fold change of 4.5 and compared to the

morula with a factor of 5 indicates that this enzyme could play an important role in the ICM cells of the rat blastocyst.
Interestingly, in the rat study the transcription factor *Stat3* was not identified in the list of genes differentially regulated with a fold change of at least of 1.5. However, we identified the genes *Stat2* and *Stat6*, the former had a fold change 1.9 and 1.6 in the comparisons B vs M and ICM vs M, the latter *Stat6* had a fold change respectively of -2.1 and -2.4 (Supplemental Table 2B and 2C). Thus, *Stat2* is upregulated in the blastocyst and in the ICM whereas *Stat6* has a higher expression in the morula. The STAT proteins are a group of transcription factors that transmit signals from the extracellular surface of cells to the nucleus and they are involved in growth, differentiation, apoptosis, and carcinogenic transformation of the cells. Interestingly, in previously performed microarray studies with mouse preimplantation embryos, *Stat2* was shown to be upregulated in the 8-cells stage embryo and then downregulated in the blastocyst stage. The *Stat6* indeed, was found upregulated in the blastocyst and downregulated in the 8-cells stage (Zeng et al., 2004). We found that in the rat embryo the expression of these two genes showed exactly the opposite behavior.

In a second step we performed a global analysis of these datasets with the GeneGo software using Metacore annotation database to assign functional biological processes to each individual species dataset. For every comparison we selected the 20 more significant processes present in the 1.5 fold change gene groups (Supplemental Fig. 1). This analysis highlighted that in the three comparisons there are different biological processes taking place in the two species. Seen that the rat preimplantation embryo development is shifted compared to the mouse of about 24 hours, it is reasonable to assume that processes like cell cycle or proliferation differs in the two species at these developmental stages (Supplemental Fig. 1).
In summary, by comparing the gene expression in the morula and blastocyst from the mouse and from the rat, we demonstrated that there are differential regulations of factors between the two species. Further analyses are needed in order to understand if these genes could have a function in the establishment of ESCs. Of special interest are those upregulated in the comparison ICM vs B in the mouse and in the rat, because they might represent new factors involved in the establishment and maintenance of the ICM cells, and therefore they might be as well critical factors in the ESCs.

Cross species analysis of selected pathways

The purpose of this study was to identify molecular pathways or genes, which are differentially expressed between the mouse and the rat, in order to gain insight into the molecular processes governing pluripotency in the rat. We analyzed fold changes between mouse and rat in 11 selected pathways from GeneGo (GeneGo Maps). A list of all the genes and the selected pathways as well as the gene fold changes is reported in the Supplemental Table 3. For every comparison (B vs M, ICM vs B, and ICM vs M) we generated a plot comparing the fold change value of the selected genes in the mouse with the ones of the same gene in the rat. Every dot represents a gene. Red dots correspond to genes that have similar fold change values between the two species. Green dots label genes with different fold change values between rat and mouse in the selected comparison. Interesting genes are highlighted with a special label that allows following the expression through all the three comparisons. This representation depicts some interesting genes with different expression pattern in the two species.

The Notch pathway

The Notch pathway is a highly conserved cell signaling system present in most multicellular organisms; it influences differentiation, proliferation, and apoptotic events at all stages of development. The Notch proteins are situated on the cell membrane and most of the ligands are transmembrane proteins, therefore the Notch pathway is important for the cell-cell communication, where the signaling is trigged only from direct cell-to-cell contact. Notch controls the cell fate choices depending on the differential expression of ligands and receptors in apposing cells, and the pathway has been implicated in different aspects of stem cell biology.
We analyzed 27 genes present in the pathway “Development Notch Signaling Pathway” in GeneGo. In the comparison B vs M most of the 27 genes behaved similar in both species and where therefore marked in red (Fig. 3A). Our study however identifies *Notch1*, one of the four known Notch receptors,

being upregulated in the mouse and downregulated in the rat in the comparison ICM vs B as well as in ICM vs M (Fig. 3A). Thus, *Notch1* is upregulated in the mouse ICM but downregulated in the rat ICM (Fig. 3B). Activated Notch1 can promote, depending from the context, either differentiation processes or maintenance of stem cell proliferation (reviewed in (Artavanis-Tsakonas et al., 1999; Kadesch, 2004)). Due to its variety of molecular functions, the finding that *Notch1* is differentially expressed in the mouse and rat preimplantation embryos could indicate a different role in the two species.
We also observed significant changes in the expression levels of other 5 genes: *Aph1a*, *Jag1*, *Maml1*, *Tle2*, and *Tle4* (Fig. 3B). The gene *Aph1a* (anterior pharynx defective) was upregulated in the mouse but only slightly changed in the rat in the comparison B vs M (Fig. 3A and Supplemental Table 3). This gene encodes the membrane protein APH-1 that is an essential member of the γ-secretase, which cleaves single-pass transmembrane proteins at residues within the transmembrane domain. The γ-secretase plays a critical role also in processing the Notch proteins (Francis et al., 2002). Moreover, it has been shown that APH-1a is the major mammalian APH-1 isoform required for Notch signaling during embryogenesis (Ma et al., 2005).
Interestingly, the Notch ligand *Jag1* (Jagged1) showed an opposite expression pattern compared to the one observed for the *Notch1* receptor (Fig. 3A). *Jag1* had fold change in the comparison B vs M of -1 for the mouse and -3.4 for the rat. This indicates that *Jag1* is highly expressed in the morula cells in both species but this upregulation is much stronger in the rat than in the mouse (Fig. 3B).
The gene *Maml1* (Mastermind-like 1) is involved in the regulation of the transcriptional activation of Notch target gene expression. *Maml1* was strongly upregulated in the mouse in the comparison B vs M and ICM vs M (Fig. 3A). In the rat however, *Maml1* was not changed in the comparison B vs M but showed an upregulation in both the comparisons ICM vs B and ICM vs M, indicating a higher expression in the cells of the ICM (Fig. 3B).
The most important targets of the Notch1-complex are the HES (hairy and enhancer of split) genes, which are transcriptional repressors that rely on the general corepressor Groucho/Transducin-like enhancer of split (TLE) protein family (Giagtzoglou et al., 2003). Thus, TLE corepressors represent a key effecter of the Notch pathway. We found that in the comparison B versus M, *Tle2* and *Tle4* have a similar expression patterns in the rat and in the mouse (Fig. 3A). However, in both the comparisons ICM vs B and ICM vs M *Tle2* and *Tle4* were downregulated in the rat, indicating a lower expression in the ICM cells (Fig. 3B).
In our study we showed that the expression of important factors like *Notch1*, *Tle2* and *Tle4* differs in the mouse and in the rat in the three cell populations analyzed. This leads to the assumption that the signaling pathway is differentially activated in the two species.

Analysis of regulators of the cell cycle

As previously mentioned there are strong differences during the preimplantation development of mouse and rat embryos. Mouse embryos need around three days to reach the blastocyst stage, what leads to a mean cell division time during this period of about 14h (Nagy et al., 2003). In reality every cell division cycle during the preimplantation development has different lengths (reviewed by (Ciemerych and Sicinski, 2005)). Of especial importance is the generation at the morula stage of blastomeres, which differ in size and cell division dynamic, and at the blastocyst stage they differentiate into trophoblast and the ICM cells. A typical characteristic of ESCs, isolated from the ICM, is that they exhibit an exceptional cell cycle distribution, where the S phase represents about 75% of the total cell cycle and the G1 phase last for about 1h (Savatier et al., 1994; Savatier et al., 1996). In the rat the formation of the blastocyst is almost 24h delayed compared to the mouse, the reason why the rat blastomeres are dividing slower than the mouse ones is largely unknown. In order to elucidate the events linked with cell cycle progression in both species we analyzed 11 genes of the GeneGo pathway "Cell cycle Influence of Ras and Rho proteins on G1/S Transition" that clearly showed differential expression in the three cell populations (Supplemental Table 3).
The genes *cyclin D1* (*Ccnd1*) and *cyclin E1* (*Ccne1)* showed different expression pattern in the mouse and the rat preimplantation embryos. The *Ccnd1* was downregulated for the mouse and upregulated for the rat in both the comparisons B vs M and ICM vs M (Fig. 4A). Thus, *Ccnd1* is in the rat predominantly expressed in the ICM cells of the blastocyst and in the mouse in the cells of the morula (Fig. 4B). CCND1, in complex with CDK4/6, phosphorylates during the S phase transition the product of the retinoblastoma (Rb). Rb is involved in the initiation of DNA replication through the activation

of E2F, which in turn activates the transcription of *Ccne1* (Harbour and Dean, 2000). The expression of *Ccne1* in the mouse showed a similar pattern like *Ccnd1*, although the downregulation was more remarkable (Fig. 4A). This indicates that in the mouse there is a predominant expression of both cyclins in the morula cells than in the blastocyst cells. In the rat however, only *Ccne1* was upregulated in the cells of the morula (Fig. 4B). Thus, both cyclins seems to have different expression pattern in the two species in the three analyzed cell populations. As mentioned before Rb is involved in the transcription of *Ccne1*. We observed an upregulation of *Rb* in the mouse for the comparisons B vs M and ICM vs M and a downregulation in the comparison ICM vs B (Supplemental Fig. 2A), indicating an increase in *Rb* expression from the morula stage to the blastocyst stage (Supplemental Fig. 2B).
Skp2 (S-phase kinase-associated protein 2) is a component of the ubiquitin ligase complex SCF, which is responsible for the ubiquitin-dependent degradation of regulators of the cell cycle. Precisely, Skp2 is involved in the degradation of the Cyclin-dependent kinase (Cdk) inhibitor p27 (Carrano et al., 1999), inducing therefore cell cycle progression. p27 prevents cell cycle progression by inhibiting the Cyclin E-Cdk2 complex formation; in the presence of the Skp2-SCF complex p27 is degraded leading to the activation of the Cyclin E-Cdk2 complex, which causes the entrance into the S phase.
The expression of *Skp2* was for the mouse downregulated in both the comparisons B vs M and ICM vs M (Fig. 4A) showing a similar expression trend like *Ccne1*: Higher in the morula cells, low in the whole blastocyst, and very low in the ICM cells (Fig. 4B). Interestingly, the expression of *Skp2* in the rat was higher in the cells of the ICM and lower in the whole blastocyst, highlighting a possible different role (Fig. 4B).
During mitosis the cells undergo profound changes in the microfilament structure. The myosin regulatory light chains (Myls) control these morphological changes through their phosphorylations (Moussavi et al., 1993; Somlyo and Somlyo, 1994). The phosphorylation of Myls is controlled by the myosin light chain kinases (Mylks). It has been shown that the Rho kinases (ROCK) are also involved in the phosphorylation of Myls (Amano et al., 1996; Kimura et al., 1996). The phosphorylation sites on the Myls vary during the cell cycle progression, inducing their activation or inhibition (Totsukawa et al., 1999; Yamakita et al., 1994). Interestingly, the expression of *Myl9, Mylk, Mylk3,* and *Rock2* was differentially regulated within the three comparisons in both species (Fig. 4A and 4B), demonstrating once more essential differences between mouse and rat preimplantation development.

In the analysis of the pathway "Cell cycle Influence of Ras and Rho proteins on G1/S Transition" we identified two members of the phosphoinositide-3-kinase pathway (PI3K-AKT): The regulatory subunit 1 (*Pik3r1*) and 3 (*Pik3r3*). Interestingly, in the rat both genes were specifically downregulated in the cells of the ICM (Fig. 4A and 4B). The PI3K-AKT pathway has been implicated in many cellular processes like regulation of cell cycle progression, apoptosis, migration, and cell adhesion. We performed the cross species analysis on the pathway "Development Growth hormone signaling via PI3K/AKT and MAPK cascades" from GeneGo (Supplemental Fig. 3A), where we analyzed the expression of *Pik3r1* and *Pik3r3* together with other members of the PI3K-AKT pathway (Supplemental Fig. 3B).
In the cross species analysis we examined the expression level of the proto-oncogene *c-Myc*. c-MYC plays important roles in various physiological processes like cell growth, proliferation, apoptosis, and loss of differentiation. In the comparisons B vs M and ICM vs M *c-Myc* was downregulated in both species, however in a more remarkable manner in the rat (Fig. 4A). Interestingly, the expression of *c-Myc* was downregulated in the rat ICM, while it was expressed at equal levels in the mouse blastocyst (Fig. 4B). This is interesting, since *c-Myc* represents an important factor in stem cell biology; moreover it is able *in vitro* in combination with three other transcription factors (*Oct3/4*, *Sox2*, and *Klf4*) to reprogram differentiated cells into pluripotent cells (Takahashi and Yamanaka, 2006).
The expression of *Gsk3β* (Glycogen synthase kinase 3 β) was found similarly regulated in both species (Fig. 4A). Nevertheless, in the rat *Gsk3β* was specifically downregulated in the cells of the ICM (Fig. 4B). It has been shown that authentic rat ESC can be derived and maintained in culture only in the presence of a GSK3β inhibitor (Buehr et al., 2008). On the contrary, pluripotent mouse ESCs can be established and maintained also under other culture conditions (Smith et al., 1988; Ying et al., 2008; Ying et al., 2003). Our data report a downregulation of *Gsk3β* in the cells of the ICM in the rat but not in the mouse, letting assume that a low level of *Gsk3β* is fundamental in the rat for maintaining the pluripotent state *in vivo* as well as *in vitro*.

Another important signaling that influences the cell cycle is the p53 pathway (Supplemental Fig. 2A and 2B). Interestingly, the gene *p53* (known in the mouse as *Trp53* and in the rat as *Tp53*) was upregulated in the rat in both the comparisons ICM vs M and B vs M (Supplemental Fig. 7A), whereas in the mouse the expression was constant in all the three cell populations (Supplemental Fig. 7B). This could explain why in the rat the gene *Nqo1* (responsible for the degradation of p53) was strongly upregulated in the ICM (Fig. 2F and Supplemental Table 2A). Other genes involved in the regulation of cell proliferation are reported in the Supplemental Figure 4, where we performed the cross species analysis on the pathway "Development SSTR2 in regulation of cell proliferation" from GeneGo.

During embryo development, the proliferation kinetics of the cells affects their fate determination, so that different cell lineages show faster or longer cell cycle progression (Ciemerych and Sicinski, 2005). Also in the ESCs *in vitro* a rigorous regulation of the cell cycle is fundamental for the maintenance of pluripotency. This study made apparent that critical factors involved in the cell cycle and proliferation are differentially expressed in the morula and the blastocyst of mouse and rat. These genes could therefore increase the list of players involved in the maintenance of pluripotency in the ESCs from both these two species.

The TGF and the Wnt signaling

The pathways transforming growth factor β (TGF-β) and Wingless (Wnt) are critically involved during the early embryo development. We analyzed 112 genes present in the pathway "Cytoskeleton remodeling TGF, WNT and cytoskeletal remodeling" from GeneGo. We highlighted 8 genes, which had a clear differential expression between the three comparisons and between the two species (Fig. 5A and 5B).

The Wnt pathway plays crucial roles in controlling genetic programs of embryonic development and adult homeostasis. Wnt signals are transduced depending on their functions through different receptors and members: The canonical Wnt pathway is known to be involved in transmitting signals for cell fate determination, whereas the non-canonical Wnt pathway is involved in controlling cell movements and tissue polarity. The gene *caveolin 1* (*Cav1*) was downregulated in the blastocyst and ICM cells of the mouse (Fig. 5A), whereas it was almost not expressed in the rat cells (Fig. 5B). Cav1 is an essential component of the caveolae, where it acts as a regulator of caveolae-dependent lipid trafficking and endocytosis (Drab et al., 2001; Nabi and Le, 2003). Cav1 can act as a positive as well as a negative regulator of important signaling pathways (reviewed in (Shatz and Liscovitch, 2008)), for example it negatively regulates the Wnt pathway by recruiting β-catenin and therefore blocking the transcription of the β-catenin target genes (Galbiati et al., 2000). The two membrane receptors *frizzled homolog 4* (*Fzd4*) and *frizzled homolog 5* (*Fzd5*) were upregulated in our analysis in the mouse ICM and blastocyst compared to the morula (Fig. 5A). However, in the rat we detected a very low expression of both receptors in all the three cell populations (Fig. 5B). This indicates that the Wnt pathway is differentially active in the two species (see also Supplemental Fig. 5). The gene *Axin2* is a downstream target of the Wnt pathway that acts as a negative regulator by directing β-catenin for proteasomal degradation (Jho et al., 2002). It has been shown that stable β-catenin and elevated *Axin2* transcription indicates the activation of the Wnt pathway (Lustig et al., 2002). In our cross species analysis *Axin2* was upregulated in the mouse in both the comparisons B vs M and ICM vs M (Fig. 5A), indicating a higher expression in the cells of the blastocyst and of the ICM (Fig. 5B). Interestingly, in the rat the expression of *Axin2* decreased specifically in the cells of the ICM (Fig. 5B). The three regulators of the Wnt pathway, namely *β-catenin* (Supplemental Fig. 2B), *Axin2* (Fig. 5B), and the *Gsk3β* (Fig. 4B) had a similar expression pattern in the rat embryos: A reduced expression in the ICM cells compared to the morula and whole blastocyst cells. In the mouse embryos the expression of these three factors was almost constant except for *Axin2* that was upregulated in the ICM and blastocyst compared to the morula.

The expression of the serine/threonine kinase *mTOR* (mechanistic target of rapamycin), which controls many biological and cellular processes such as cell growth, proliferation, and different types of differentiation (Hay and Sonenberg, 2004; Wullschleger et al., 2006), was upregulated in the mouse ICM (Fig. 5A) but showed a decreasing expression in the rat from the morula to the blastocyst stage (Fig. 5B).

The Wnt and TGF pathways control fundamental processes during the embryo development, and here we highlighted that the regulation of these pathways differ between the mouse and the rat. Interestingly, analysis of the expression of genes involved in the apoptotic and survival processes revealed as well differential expression between the two species. We analyzed 13 genes from the pathway "Apoptosis and survival NGF signaling pathway" (Supplemental Fig. 6A and 6B) and 20 genes from the pathway "Apoptosis and survival Apoptotic Activin A signaling" (Supplemental Fig. 7A and 7B) from GeneGo. For example the apoptosis related gene *Caspase3* (*Casp3*) was upregulated in the rat in all the three comparisons (Supplemental Fig. 6A) indicating a higher expression in the cells of the blastocyst (Supplemental Fig. 6B). On the contrary in the mouse, *Casp3* was upregulated in the cells of the morula and then the expression decreased in the blastocyst (Supplemental Fig. 6B).

In summary, this study identified within important signaling pathways interesting candidates differentially expressed in the mouse and in the rat preimplantation embryos. We believe that these differences observed *in vivo* might reflect also the differences observed between the mouse and the rat ESCs, for instance in their derivation efficiency and maintenance. Further analyses are needed in order to clarify which roles do they have in ESCs, and furthermore if they might represent new pluripotency factors.

Cross species analysis of the expression patterns of selected gene families

Based on the genes present on GeneChip® Mouse Genome 430 2.0 arrays and, for the rat on the GeneChip® Rat Genome 230 2.0 arrays, we selected the families of genes. With the same approach used for the analysis of the pathways in the morula and in the blastocyst stage, we further characterized the expression pattern of the genes in the three cell populations for the mouse and for the rat. The complete list of the selected families of genes as well as the fold changes in the three comparisons are listed in the Supplemental Table 4.

The BMP-ligands and -receptors family with the intracellular SMADs-family

The bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF) super-family and are involved in a variety of processes during embryo development like in the generation and maintenance of organs, in which stem cells play important roles. The signaling pathway starts when the secreted BMP proteins bind to the type I and type II BMP receptors, inducing the activation of the intracellular substrates, the SMAD proteins.
Here we analyzed the expression of 10 BMP proteins, 4 BMP receptors, and 6 SMAD proteins in the morula, the blastocyst and the isolated ICM, from the mouse and from the rat (Supplemental Table 4). The genes *Bmp15* and *Bmp4* showed in both species the same expression pattern: Being the former downregulated and the latter upregulated in both the comparisons B and ICM vs M (Fig. 6A). This indicates that *Bmp15* is prevalently expressed in the cells of the morula whereas *Bmp4* is upregulated in the cells of the blastocyst and ICM. It is interesting to note that *in vivo* the pluripotent cell population (ICM) of the rat and the mouse has a similar expression of *Bmp4*. It has been shown that *in vitro*, murine ESCs can be maintained in serum-free culture in the presence of BMP4 or BMP2 in combination with LIF (Ying et al., 2003). Nevertheless, withdrawal of LIF and retention of BMP4/2 causes differentiation into epithelial-like cells, leading to the conclusion that the self-renewal response to BMP is dependent on continuous LIF signaling and that the BMP main function is to antagonize the neural differentiation induced by LIF in the absence of serum (Ying and Smith, 2003). All the attempts to derive rat ESCs in serum-containing medium failed in the last years so that nowadays it is possible to establish rat ESCs only under defined, serum-free conditions (Buehr et al., 2008). Taken together, seen that the expression of *Bmp4* in the ICM of the mouse and the rat blastocyst is similar, it would be interesting to analyzed the role of BMP4 in rat ESC derivation and maintenance. The expression analysis of other *Bmp*-ligands revealed a general upregulation in the mouse and downregulation in the rat (Fig. 6A) whereas no major differences in the two species could be observed for the *Bmp* receptors (Fig. 6B). In the comparison ICM vs M the sole gene that was differentially expressed between mouse and rat was *Bmpr1a* that was upregulated in the rat but did not have differential expression in the mouse (Fig. 6B).

In the BMP signaling pathway the activated receptors recruit the SMAD molecules, which transmit the signal from the cell surface to the nucleus (Supplemental Table 4). The expression of the receptor-regulated *Smad1, -2, -3* had a similar expression pattern in both the species in all the three comparisons (Fig. 6C). The products of these genes are transcription factors that form complexes with SMAD4 and regulate gene transcription. The expression of *Smad4* was found for the mouse higher in all the cells of the blastocyst than in the morula (Fig. 6C) whereas in the rat expression of *Smad4* persisted from the morula to the blastocyst but was specifically upregulated in the cells of the ICM (Fig. 6C). SMAD6 and SMAD7 are both negative regulators of the BMP pathway. They bind to the receptor-regulated SMADs and block their ability to interact with the Co-SMAD (SMAD4). In the comparisons ICM vs B and ICM vs M the expression of *Smad7* was in both cases downregulated in the mouse but upregulated in the rat cells (Fig. 6C). It is interesting to notice, that in the mouse we observed an upregulation of the transcription factors *Smad3* and *Smad2* in the cells of the ICM and the blastocyst, together with an upregulation of the Co-regulator *Smad4*, whereas the expression of *Smad7* is specifically downregulated in the ICM (Fig. 6E).
Analysis of the pathway called "Development BMP signaling" from GeneGo revealed that other genes involved in this pathway are differentially regulated in the morula, ICM, and blastocyst of the mouse and the rat (Fig. 6D). The BMP pathway plays important roles in the differentiation of ESCs *in vitro*. Rat ESCs seems to be more sensitive to differentiation stimuli than mouse ESCs, therefore the differential regulation observed *in vivo* of the factors involved in this pathway might reflect also a differential expression *in vitro*, in mouse and rat ESCs.

The FGF-factors and FGFR-receptors family

The fibroblast growth factor (FGF) ligands and receptors have been implicated in different phases of the early embryogenesis (Goldfarb, 1996). The FGF signaling controls proliferation and differentiation of the cells, cell survival, cell morphology and migration, through the activation of important cytoplasmic signal transduction pathways like for example the Ras/ERK pathway and the AKT pathway (Dailey et al., 2005; Mohammadi et al., 2005).
We analyzed the expression in the three cell populations of 21 FGF factors and 7 cell surface FGF receptors present on the mouse and the rat microarray chip (Supplemental Table 4). The expression of *Fgf4* was constant in the mouse morula and blastocyst, in the rat embryos however, *Fgf4* expression was upregulated in the comparison B vs M and downregulated in the ICM vs B (Fig. 7A). Thus, the expression of *Fgf4* in the rat preimplantation embryo is low in the ICM cells but higher in the trophoblast cells of the blastocyst. This observation is interesting, since it was reported that in the mouse embryo the expression of *Fgf4* was first detected in the whole blastocyst (Niswander and Martin, 1992) and its expression persists also *in vitro*, in murine ESCs. A recent study reported the generation of trophoblast stem (TS) cells from rat blastocysts (Asanoma et al., 2011). Mouse TS cells are generated from 3.5 blastocyst stage embryos and are maintained and cultivated *in vitro* through the activation of the factor FGF4/FGF receptor 2 (Tanaka et al., 1998). Rat TS cells share many similarities with the mouse TS cells, like the FGF4-dependance (Asanoma et al., 2011). Nevertheless, rat TS cells showed a differential expression of some important markers compared to the mouse cells. The authors concluded that the rat TS cell population isolated from E4.5 blastocysts, show features more developmentally advanced in comparison to the TS cell population isolated from mouse blastocysts (Asanoma et al., 2011). The fact that we observed a higher expression of *Fgf4* in the rat blastocyst and specifically in the trophoblast cells, compared to the mouse, could explain the differences observed in the derivation of TS cells in the two species.
The family of the FGF receptors consists in four genes: *Fgfr1, -2, -3, -4*. After alternative splicing they generate a variety of receptors with differential affinities for their ligands (Zhang et al., 2006). The gene *Fgfr4* was in the mouse downregulated in both the comparisons ICM vs B and ICM vs M, indicating an expression in the morula and trophoblast cells of the blastocyst (Fig. 7B), its expression was however not changed in the rat cell populations. The expression of *Fgfr2* increased for both species in the blastocyst, although the upregulation was more predominant in the rat than in the mouse (Fig. 7B). The analysis of the pathway "Development FGFR signaling pathway" from GeneGo also highlighted differential expression patterns of genes in the two species (Fig. 7C). For example the expression of the gene *Raf1* (*v-raf-leukemia viral oncogene 1*) was similar in the cells of the morula in the mouse and in the rat. However, for the mouse it was downregulated in the ICM cells and

upregulated in the whole blastocyst, whereas for the rat it was upregulated in the ICM and downregulated in the whole blastocyst (Fig. 7D). Raf1 is a member of the MAPK/ERK pathway (mitogen-activated protein kinase/extracellular receptor kinase), which is stimulated by the FGF factors during embryo development. In a previous study the expression of *Raf1* was detected in both the ICM cells and the trophoblast cells of the mouse blastocyst in a similar amount (Wang et al., 2004). We measured however, a downregulation of *Raf1* expression in the ICM cells and an upregulation in the trophoblast cells of the blastocyst. This is in agreement with its involvement in the activation of the FGF signaling that is responsible for the maintenance of the trophoblast cells. Interestingly, the expression of *Raf1* in the rat was downregulated in the trophoblast cells (Fig. 7D) and upregulated in the ICM cells, leading to the assumption that this member of the MAPK pathway plays a role in the ICM cells of the rat blastocyst. The family of MAPKs consists in serine/threonine kinases that play essential roles in signal transduction and are involved in a variety of biological processes. We analyzed 13 members of this family and we found differences in the expression of several genes in the three cell populations of the mouse and the rat (Supplemental Fig. 8A and Supplemental Table 4).

The Wnt-ligands family

We have already reported some important changes in the expression of members of the Wnt pathway (Fig. 5 and Supplemental Fig. 5). Wnt genes encode a large family of secreted, cysteine-rich proteins that play key roles as intercellular signaling molecules in developmental processes. Here we analyzed 17 members of the Wnt-secreted factors and interestingly, we observed that the expression of many Wnt genes is differentially regulated in the mouse and in the rat (Supplemental Table 4). For example *Wnt6* was upregulated in the trophoblast cells of the mouse blastocyst whereas it was upregulated in the cells of the morula in the rat embryos (Fig. 8A). The opposite expression pattern was observed for the gene *Wnt4*, that was upregulated in the mouse in the morula and in the rat in the blastocyst cells. Interestingly, in the rat *Wnt5a* was highly expressed in the cells of the morula and in a lesser extend in the ICM cells (Fig. 8A), whereas in the mouse its expression showed only minor differential regulation between the three comparisons (Supplemental Table 4). The role of the Wnt5a ligand has been extensively studied since it acts through both the canonical and non-canonical Wnt pathway (Mikels and Nusse, 2006). Importantly, the canonical Wnt pathway has been implicated in the maintenance of pluripotency in murine ESCs. The WNT5A ligands, together with WNT6, WNT3, and WNT3A were reported to be sufficient for maintaining murine ESCs in an undifferentiated state in the absence of LIF (Hao et al., 2006). Although the precise mode of action of the Wnt pathway in maintaining pluripotency in ESCs needs still to be clarified, it is important to note that factors like *Wnt5a* and *Wnt6* are differentially regulated in the mouse and in the rat in the pluripotent cell compartment of the blastocyst (Fig. 9A).

The Stat family

The Signal transducer and activator of transcription (STAT) proteins are cytoplasmic transcription factors that transmit the information received from the transmembrane receptors directly to the nucleus of the cells, where they target the promoter of genes involved in survival, proliferation, and differentiation (Frank, 2007). Here we analyzed the expression of 5 members of the STAT family (Fig. 8B and Supplemental Table 4). The type I interferons (IFN) are involved in antiproliferative, apoptotic, and antiviral processes, and they are responsible for the activation of STAT1 and STAT2 (Fu et al., 1992). In the rat *Stat2* was upregulated in the blastocyst cells, however in the mouse *Stat2* was upregulated in the cells of the morula compared to the blastocyst (Fig. 8B). The S*tat6* expression was upregulated in the rat in the cells of the morula, whereas it did not show differential expression in the mouse cell populations (Fig. 8B).
In the comparison ICM vs B all the *Stats* showed a similar expression in the mouse and in the rat. Only *Stat5a* and *Stat5b* were differentially regulated, being the former higher expressed in the trophoblast cells of the mouse blastocyst whereas the latter was upregulated in the trophoblast cells of the rat blastocyst (Fig. 8B).

This analysis showed that members of the *Stat* family are differentially regulated in the mouse and rat preimplantation embryos, advising a possible different implication in the development of the morula and blastocyst in the two species.

Expression pattern analysis of genes related to pluripotency

The goal of this study was to give a general overview on the regulation of the molecular mechanisms that take place during the development of the mouse and the rat preimplantation embryo form the morula to the blastocyst stage, in order to highlight similarities and differences that could help in the derivation and maintenance of rat ESCs. The LIF/gp130 pathway that leads to the activation of the transcription factor STAT3, plays a fundamental role in the maintenance of pluripotency in mouse ESCs (Casanova et al., 2011b; Cinelli et al., 2008; Matsuda et al., 1999; Niwa et al., 1998; Smith et al., 1988) as well as in rat ESCs (Buehr et al., 2008; Li et al., 2008). Controversially, although ESCs show LIF dependence (under certain culture conditions), early epiblast cells do not require LIF stimulation. In fact, *Lif* -/- embryos develop into later stages (Stewart et al., 1992) and embryos carrying mutations on the LIFβR and gp130 receptor develop normally, at least until mid-gestation (Li et al., 1995; Nakashima et al., 1999). Nevertheless, the LIF/STAT3 pathway is indispensable during the preimplantation development, in case of diapause (Nichols et al., 2001). This observation could explain why embryos do express all the component of this pathway and moreover, why ESCs that are directly derived from the ICM of the blastocyst, are LIF-dependent (reviewed in (Graf et al., 2011)). Due to the importance of the LIF/gp130-STAT3 pathway in the maintenance of pluripotency in ESCs, we selected 11 genes involved in this pathway and we analyzed their expression in the mouse and rat morula, blastocyst, and ICM.
Interestingly, the expression of *Lif* increased in the mouse from the morula to the blastocyst, having a lower expression in the cells of the ICM. On the contrary, in the rat its expression was stable in the ICM cells as well as in the whole blastocyst (Fig. 9A). A behavior similar in the two species was observed for *Jak2* (Janus kinases2) that was specifically downregulated in the ICM but upregulated in the blastocyst (Fig. 9A). *Jak1* expression indeed, showed in the mouse an analog expression pattern like *Lif*, whereas in the rat it was specifically downregulated in the cells of the ICMs (Fig. 9A). The binding of the cytokine LIF to the receptor results in the heterodimerization of the LIFβR and gp130 that causes the activation of receptor-associated JAKs, which are responsible for the phosphorylation and activation of STAT3. JAK1 is necessary for the transmission of the LIF-induced signaling, whereas JAK2 is dispensable. Thus, due to the higher LIF-dependence of rat ESCs in comparison to murine ESCs, it would be of interest to analyze the expression of *Jak1* in rat ESCs. Interestingly, also the expression of *Stat3* was reduced in the rat ICM cells compared to the whole blastocyst, whereas in the mouse it was constant. Nevertheless, at the morula stage both mouse and rat showed a similar expression level of *Stat3* (Fig. 9B). The transcription of the *Socs* genes (Suppressor of cytokine signaling) is directly controlled by STAT3. *Socs3* is responsible for the negative regulation of the LIF/STAT3 signaling (O'Sullivan et al., 2007). Although we observed a general upregulation in the mouse preimplantation embryo of the components of the LIF pathway, the expression of *Socs3* was downregulated in the ICM and in the whole blastocyst (Fig. 9B). Interestingly, in the rat embryos *Socs3* expression increased in a similar manner like *Stat3*, from the morula to the blastocyst stage (Fig. 9B) suggesting again that a well-balanced LIF/STAT3 activation is crucial in the rat.
In parallel to the activation of the STAT3 pathway, binding of LIF to the LIFβR/gp130 receptor leads the activation of the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3 phosphate kinase (PI3K) pathways. Active gp130 receptor can associate with the protein tyrosine phosphatase SHP-2 (Fukada et al., 1996), which leads to the activation of the kinases RAS/RAF and finally ERK1/2. The expression of *Shp2* was specifically downregulated in the rat ICM cells whereas it was upregulated in the mouse ICM (Fig. 9C). However, the expression of *Raf1* had exactly the opposite expression pattern: Downregulated in the mouse ICM cells and upregulated in the rat ICM, indicating a differential expression in both the ICM cells and the trophoblast cells in the two species (Fig. 9C). ERK regulates early differentiation processes *in vivo* as well as *in vitro* (Kunath et al., 2007; Nichols et al., 2009b), so that it has been shown that inhibition of this pathway together with the inhibition of GSK3 is sufficient for maintaining pluripotency in ESCs in the absence of LIF (Ying et al., 2008).

A downstream effector of the PI3K pathway is the serine/threonine protein kinase B (PKB, also known as AKT). AKT has been implicated in many cellular processes like regulation of the cell cycle progression, cell death, adhesion, migration, metabolism and tumorigenesis. In the mouse and in the rat preimplantation embryo we observed a similar expression pattern of *Akt1*, which increased from the morula to the blastocyst stage, although in the mouse the increase was more prominent (Fig. 9C).
The genes *Sox2*, *Klf4*, and *Klf2* are involved in ESCs in the maintenance of pluripotency (Hall et al., 2009; Masui et al., 2007; Niwa et al., 2009). Moreover, *Sox2* and *Klf4* together with *cMyc* and *Oct3/4* are the four factors used for reprogramming differentiated cells into induced pluripotent stem cells (iPSC) (Takahashi and Yamanaka, 2006).
SOX2 is a member of the sex-determining region of the Y chromosome-related (SRY-related) high-mobility group (HMG) box (SOX) family of transcription factors. *Sox2* expression is downregulated in cells with restricted developmental potential. We observed an upregulation of *Sox2* expression in the mouse from the morula to the blastocyst stage (Fig. 9D). Interestingly, in the rat embryos *Sox2* was expressed in a lower level compared to the mouse; moreover it was slightly downregulated in the blastocyst compared to the morula (Fig. 9D).
Some of the *Klf* genes (Kru¨ppel-factors) have been proposed as downstream targets of LIF/STAT3 pathway in ESCs (Hall et al., 2009; Niwa et al., 2009). In our analysis we observed that *Klf4* expression increased in the cells of the rat ICM and was downregulated in the whole blastocyst, whereas in the mouse embryos the upregulation of *Klf4* was less strong in the ICM cells (Fig. 9D). Also *Klf2* in the rat was upregulated in the ICM and blastocyst but it was downregulated in the mouse blastocyst and ICM cells (Fig. 9D). This is interesting since *Klf2* and *Klf4* have been implicated with important pluripotency factors in murine ESCs (Hall et al., 2009). Thus, the fact that they are differentially regulated in the morula and blastocyst from the rat compared to the mouse, could be a contributing factor for the differences observed between murine and rat ESCs in the derivation efficiency and culture conditions.

The higher genetic diversity of the rat compared to the mouse (Canzian, 1997) has made the rat an optimal animal model for the investigation of human diseases, such as infectious and autoimmunity diseases, or for toxicology and drug development. Moreover, the rat has other advantages compared to the mouse like for instance the bigger size or the higher learning capacity that make it a convenient research animal model. Nevertheless, the impossibility for many years to generate authentic rat ESCs has give the mouse a clear advantage over the rat as a model for biomedical research.
With this study we aimed at the identification of differences at the transcriptional level between the mouse and the rat during the embryo development in which the ICM cells are formed, since they represent the source of ESCs derivation. The differential regulation of critical genes could represent the starting point for analyzing their function *in vitro* in mouse and rat ESCs. Furthermore, this knowledge could be critical for the improvement of the derivation and maintenance of rat ESCs. Although recently rat ESCs have been generated (Buehr et al., 2008; Li et al., 2008) there are still many questions open. A broader knowledge on the molecular mechanisms that occur in rat ESCs would improve the efficiency of establishing stable authentic pluripotent rat ESCs and therefore it would facilitate the generation via gene targeting of transgenic rat models, which are indispensable for the biomedical research.
This is the first study that investigated the gene expression changes during the transition from morula to blastocyst in the rat preimplantation development. Moreover, our study represents a new example of statistical approach for cross species analysis that could be applicable also for other species. The so-obtained data allows highlighting the species-specific behavior of genes within important pathways and families through the creation of own gene networks. An example of network of genes that behave in a different way in mouse and rat is presented in Figure 10.
The presented genome wide cross species analysis in the three cell populations (morula, blastocyst, and isolated ICMs) highlights differential expression within the two species of important genes like β-catenin, *Bmp4*, or *Smad4*. All of them are part of essential signaling pathways that play critical functions during the embryo development, and their differential regulation strongly affects the activation or inhibition of the pathways in which they are involved. Some of the genes are also known to be important factors in the maintenance of pluripotency in ESCs, like for example *Sox2* or *Stat3*, or play a role in reprogramming somatic cells to pluripotency like *c-Myc*, *Klf4* and *p53* and would therefore represent interesting candidates to further analyze *in vitro* in the rat ESCs.

In conclusion, this study represents a good starting point for further analysis aimed at the identification of new factors related to pluripotency in both the species.

Materials and Methods

Mouse and Rat strains

Mouse: Hybrid B6D2F1 mice (female C57BL/6 x male DBA/2). Rat: Outbreed Wistar Han rats. Animals were housed under controlled lighting (lights on at 0600–1800h), temperature (23 ± 2°C) and humidity (50 ± 5%), with free access to food and water. The Veterinary Office of the Canton of Zurich, Switzerland approved all animal experiments. Housing and experimental procedures were in accordance with the Swiss animal protection law and conformed to the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Council of Europe no. 123, Strasbourg 1985).

Collection of mouse embryos

3 to 5 weeks old B6D2F1 females were superovulated with 5IU pregnant mare's serum gonadotropin (PMSG) (Folligon® Intervet) and 48h later, with 5IU human chronicle gonadotropin (hCG) (Chorulon® Intervet). Superovulated females were mated with B6D2F1 males for 24h and then housed separately to the males. At embryonic day 2.5 (E2.5) pregnant females were sacrificed and morula stage embryos were collected from the oviducts. Morula were either frozen for further RNA extraction, or cultivated over night in M16 medium (Sigma) at 37C°, under 5% CO_2 in air. Under these conditions morula developed to the blastocyst stage. Blastocyst embryos were either stored at -80°C for further RNA extraction or immunosurgery was performed for the isolation of the inner cell masses (ICMs).

Isolation of ICMs by immunosurgery with mouse blastocyst stage embryos

Zona pellucida was removed by shortly incubating the blastocysts with Tyrode's solution (Sigma). Consequently zona-free blastocysts were washed several times with M2 medium (Sigma) and immunosurgery was performed, by incubating the embryos 30min with rabbit anti-mouse serum (Sigma). Afterwards, embryos were washed several times with DMEM medium (GIBCO) supplemented with 10% of fetal bovine serum. The embryos were then incubated (37C°, 5% CO2) with guinea pig complement serum (Sigma) for another 30 min. Rabbit anti-mouse serum was used 1:30 diluted in DMEM medium; guinea pig complement serum was diluted 1:3 in DMEM. The isolated ICMs were stored at -80°C till total RNA was extracted.

Collection of rat embryos

6 to 12 weeks old Wistar females were check for estrus by using the Estrus Cycle Monitor EC40 (Fine Science Tools, Foster City, CA) as previously described (Ramos et al., 2001). All the positive females were breaded with Wistar males for 24h and then housed separately to the males. At day E4 of pregnancy Wistar females were sacrificed and morula embryos were isolated and stored for later RNA extraction. Blastocyst embryos were isolated at E4.5 and either collected for further RNA extraction or processed for immunosurgery.

Immunosurgery with rat blastocyst stage embryos

After removal of the zona pellucida, blastocysts were incubated for 3 hours (37C°, 5% CO2) in rabbit anti-rat serum (Sigma). Afterwards, embryos were washed several times with DMEM medium (GIBCO) supplemented with 10% of fetal bovine serum and incubated with rat serum (made in-house) for 20 min (37C°, 5% CO2). Rabbit anti-rat serum was used 1:5 diluted in DMEM medium; rat serum was diluted 1:5 in DMEM. The isolated ICMs were stored at -80°C till total RNA was extracted.

Total RNA extraction from mouse and rat embryos

Till the desired amount of embryos were reached, embryos were stored in RLT buffer supplemented with 1% of β-mercaptoethanol (first lyses buffer of the RNeasy Micro Kit (QIAGEN) protocol) at -80°C. Two pools of embryos for each developmental stage where formed (Fig. 1C). Total RNA was extracted by using RNeasy Micro Kit (QIAGEN) according to the manufactures' recommended protocols. All RNA samples were kept at -80°C till they were processed for microarray hybridization.

Mouse and Rat Microarray Experiment Description

cRNA preparation: The quality of the isolated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Delaware, USA) and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). The cDNA was prepared from total RNA using a primer mix and reverse transcriptase (RT) (WTOvation Pico System, NuGEN, 3300-12). The primers have a DNA portion that hybridizes either to the 5' portion of the poly (A) sequence or randomly across the transcript. SPIA amplification, a linear isothermal DNA amplification process, was used to prepare single-stranded cDNA in the antisense direction of the mRNA starting material. Single-stranded cDNA quality and quantity was determined using NanoDrop ND 1000 and Bioanalyzer 2100. Fragmented and biotin-labeled single-stranded cDNA targets were generated with the FL-Ovation cDNA Biotin Module V2 (NuGEN, 4200-12).
Array hybridization: Biotin-labeled single-stranded cDNA targets (5µg) were mixed in 220µl of Hybridization Mix (Affymetrix Inc., P/N 900720) containing a Hybridization Controls and Control Oligonucleotide B2 (Affymetrix Inc., P/N 900454). The six mouse samples were hybridized to GeneChip® Mouse Genome 430 2.0 arrays for 18h at 45°C the same procedure was used for the six rat samples that were hybridized to GeneChip® Rat Genome 230 2.0 arrays. Arrays were then washed using an Affymetrix Fluidics Station 450 FS450 0004 protocol. An Affymetrix GeneChip Scanner 3000 (Affymetrix Inc.) was used to measure the fluorescent intensity emitted by the labeled target.

Processing of microarray data

Raw data processing was performed using the Affymetrix AGCC software. After hybridization and scanning, probe cell intensities were calculated and saved in appropriate CEL files. The CEL files have been processed by scripts in R, prepared with Bioconductor (Gentleman et al., 2004) libraries: affy, simpleaffy, limma, gplots and biomaRt. Mouse CEL files have been grouped for summarization with Brainarray (Dai et al., 2005) custom CDF file Mouse4302_Mm_ENSG (16860 gene product summaries), while rat CEL files had the probes grouped by Rat2302_Rn_ENSG CDF file (11462 gene product summaries). Summarization was performed with standard RMA algorithm (Irizarry et al., 2003).

Mapping of genes between the species

The table of mouse-rat orthologs, labeled with Ensembl gene identifiers, has been prepared with biomart interface (http://biomart.org) to Ensembl database (Flicek et al., 2011) (Ensembl genes v60, mouse genome NCBIM 37, rat genome RGSC 3.4). All the mouse genes present in the Brainarray summary file have been translated to rat orthologs, resulting in the translation table that includes 13'139 mouse genes (ca 77.9% of those in the array summary) and 9'083 rat genes (79.2% of those in the array summary). The table has been used to map mouse genes to rat orthologs and *vice versa*.

Selection of gene sets from pathways and families

The pathways and the genes included in them have been extracted from Metacore database via GeneGo tool (Thomson Reuters, http://portal.genego.com, (Nikolsky et al., 2009)). For all the pathways, a list of genes with Ensembl identifiers has been extracted and the genes that have mouse-rat ortholog pairs in the mapping table have been selected. The pathways (groups of genes belonging to the same pathway map in GeneGo Metacore database) and gene families have been selected on the basis of the relevance to various developmental processes. All the analyses have been repeated for the

groups of genes belonging to the selected pathways and families. For the graphical overview of the sample processing and data analysis see Fig. 1D.

Discovering patterns of similarities and differences within groups

For all the pairs of tissues (blastocyst versus morula, ICM versus blastocyst, and ICM versus morula), the fold changes have been calculated for both: mouse and rat separately. To find the genes that have expression characteristics similar or different in each of the pairs of samples the heuristics have been defined. As similar are regarded genes with the difference of fold change smaller than a specific threshold (0.2 on log2 scale). On the scatterplots these genes are marked with red. As different are marked those that have the absolute difference of fold changes bigger than a threshold of 0.4, but also having both mouse and rat fold changes absolute value bigger than a threshold of 0.2 - to choose the genes that are not on the fold change scatterplot diagonal, but excluding the genes not differentially expressed in one of the specie. Genes that satisfy the "differential inter-species fold change conditions" are marked green. The rest of genes that do not fulfill “different” or “similar” condition, are marked plain black on the scatterplot.
“similar” pattern genes (red):

$$g : \left| FC_{mouse}(g) - FC_{rat}(g) \right| < 0.2$$

“different” pattern genes (green):

$$g : \left| FC_{mouse}(g) - FC_{rat}(g) \right| > 0.4 \wedge \left| FC_{mouse}(g) \right| > 0.2 \wedge \left| FC_{rat}(g) \right| > 0.2$$

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Figure Legends

Figure 1. Mouse and rat late preimplantation embryo development: a cross species analysis. A. Schema of mouse and rat embryo development length. M: morula stage embryo; B: blastocyst stage embryo. E: embryonic day. **B.** Schema of the three cell populations used for the cross species analysis of gene expression. The blastocyst embryo consists in the inner cell mass (ICM) and in the trophoblast cells. Embryonic stem cells (ESCs) are derived *in vitro* from the ICM cells. **C.** Numbers of embryos collected for the whole genome expression analysis. **D.** Schematic overviews of the screening strategy used in this study. M1 and M2: pool 1 and pool 2 of morula stage embryos; B1 and B2: the two pools of blastocysts; ICM 1 and ICM2: the two pools of isolated ICMs. B vs M: blastocyst versus morula; ICM vs B: ICM versus blastocyst; ICM vs M: ICM versus morula.

Figure 2. Global significant analysis. A. Mouse **B.** Rat heatmaps. The genes are selected by the highest fold change – 20 genes in each pair wise comparison of sample means. M1 and M2: morula samples 1 and 2; B1 and B2: blastocyst samples 1 and 2; ICM1 and ICM2: isolated inner cell mass (ICM) cells samples 1 and 2. **C-F.** Venn diagrams of the overlap of mouse and rat genes with significant differential expression (fold change higher than 1.5 and higher than 3) in the three comparisons: ICM versus (vs) Blastocyst, Blastocyst versus Morula, and ICM versus Morula.

Figure 3. Cross species analysis of the genes in the Notch pathway. A. Fold change scatterplots. Cross species comparison of the fold changes expression of the genes in the pathway "Development, Notch Signaling Pathway" from GeneGo. In green are marked the genes for which fold change differ in mouse and rat within the three comparisons: Blastocyst versus (vs) Morula, ICM versus Blastocyst, and ICM versus Morula. In red are marked those genes that have a similar fold change pattern in the two species in each comparison. With a special marker there are highlighted 6 selected genes that have differential expression patterns in the two species. The complete list of all the genes analyzed with their fold changes is reported in the Supplemental Table 3. **B.** Expression signal profile plots. The expression level of 6 selected genes from the Notch pathway. In blue are marked the expression levels of the genes in the mouse and in red the one in the rat embryos. MO: Morula, ICM: Inner cell mass, BL: Blastocyst.

Figure 4. Cross species analysis of cell cycle component. A. Fold change scatterplots. Cross species comparison of the fold changes expression of the genes in the pathway "Cell cycle, Influence of Ras and Rho proteins on G1/S transition" from GeneGo (see also Supplemental Table 3). The data were analyzed as described in Fig. 3A. Thirteen genes have been highlighted in order to follow their expression in the three comparisons. **B.** Expression signal profile plots. Expression level of 13 selected genes involved in the regulation of the cell cycle. In blue are marked the expression level of the genes in the mouse and in red the one in the rat embryos. MO: Morula, ICM: Inner cell mass, BL: Blastocyst.

Figure 5. Cross species analysis of the genes in the Wnt and TGF pathways. A. Fold change scatterplots. Cross species comparison of the fold changes expression of the genes in the pathway "Cytoskeleton remodeling, TGF, WNT and cytoskeletal remodeling" from GeneGo (see also Supplemental Table 3). The data were analyzed as described in Fig. 3A. Eight genes have been marked with a special label. **B.** Expression signal profile plots. Expression level of 8 genes in the morula, the ICM, and the blastocyst in the mouse (blue) and in the rat (red). MO: Morula, ICM: Inner cell mass, BL: Blastocyst.

Figure 6. Cross species analysis of regulators of the BMP pathway. A. The BMP protein family. Scatterplots of the fold changes measured in the three comparisons for 9 members of the BMP protein family in the mouse and in the rat. The complete list of all the genes analyzed as well as their fold changes are reported in the Supplemental Table 4. **B-C.** Same analysis like for the BMP proteins was performed for 4 members of the BMP receptor family (**B.**) and for 6 members of the SMAD protein

family (**C.**). **D.** Fold change scatterplots. Cross species comparison of the fold changes expression of the genes in the pathway "Development, BMP signaling" from GeneGo (see also Supplemental Table 3). The data were analyzed as described in Fig. 3A. **E.** Expression signal profile plots. Expression level analysis of 4 selected genes from the BMP pathway. Mouse: blue; Rat: red; MO: Morula; ICM: Inner cell mass; BL: Blastocyst.

Figure 7. Cross species analysis of regulators of the FGF pathway. A-B. Scatterplots of the fold changes measured in the three comparisons for 21 members of the FGF factor family (**A.**) and for 7 FGF receptors (**B.**) in the mouse and in the rat. The complete list of all the genes analyzed as well as their fold changes are reported in the Supplemental Table 4. **C.** Fold change scatterplots. Cross species comparison of the fold changes expression of the genes in the pathway "Development, FGFR signaling pathway" from GeneGo (see also Supplemental Table 3). The data were analyzed as described in Fig. 3A. **D.** Expression signal profile plots. Expression level analysis of 5 selected genes from the FGFR pathway. Mouse: blue; Rat: red; MO: Morula; ICM: Inner cell mass; BL: Blastocyst.

Figure 8. Cross species analysis of the Wnt ligands family and the STAT family. A-B. Scatterplots of the fold changes measured in the three comparisons for 11 members of the Wnt family (**A.**) and for 5 STAT family members (**B.**) in the mouse and in the rat. The complete list of all the genes analyzed as well as their fold changes are reported in the Supplemental Table 4.

Figure 9. Expression signal profile plots for 11 genes involved in the LIF/gp130 signaling. Expression signal profile plots. **A.** Expression level analysis of *Lif,* which encode the ligand that binds on the LIFβR/gp130 receptor, and *of Jak2* and *Jak1* the receptor-associated Janus Kinases involved in the propagation of the extracellular signaling. **B.** Expression level analysis of *Stat3* and *Socs3*. The transcription factor STAT3 directly controls the transcription of the negative regulator SOCS3. **C.** Expression level analysis of *Shp2*, *Raf1*, and *Akt1*. The products of these three genes lead to the activation of the ERK- and PI3K/AKT-pathways. **D.** Expression level analysis of *Sox2, Klf4,* and *Klf2*. These genes are involved in the maintenance of pluripotency in ESCs. Mouse: blue; Rat: red; MO: Morula; ICM: Inner cell mass; BL: Blastocyst.

Figure 10. Network of selected differentially expressed genes between mouse and rat in the comparison blastocyst versus morula. Network of selected genes that show different expression patterns in mouse and rat in the comparison blastocyst versus morula. The genes come from the pathway and family gene lists (Supplemental Table 3 and 4), the graph has been created with GeneGo network editor tool.

Supplemental Figures

Suppl. Figure 1. Most significantly enriched Gene Ontology Biological Process terms according to GeneGo. The lists of genes counted for the enrichment are those that have been used for the Venn diagram with 1.5 fold change differences.

Suppl. Figure 2. Cross species analysis of the genes in the p53 pathways. A. Fold change scatterplots. Cross species comparison of the fold changes of the genes of the pathway "Transcription, p53 signaling pathway" from GeneGo (see also Supplemental Table 3). The data were analyzed as described in Fig. 3A. **B.** Expression signal profile plots. Expression pattern analysis of 4 genes from the p53 pathway. Mouse: blue; Rat: red; MO: Morula; ICM: Inner cell mass; BL: Blastocyst.

Suppl. Figure 3. Cross species analysis of PI3K/AKT and MAPK cascades. A. Fold change scatterplots. Cross species comparison of the fold changes of the genes in the pathway "Development, growth hormone signaling via PI3K/AKT and MAPK cascades" from GeneGo (see also Supplemental Table 3). The data were analyzed as described in Fig. 3A. **B.** Expression signal profile plots.

Expression pattern of 6 genes. Mouse: blue; Rat: red; MO: Morula; ICM: Inner cell mass; BL: Blastocyst.

Suppl. Figure 4. Cross species analysis of factors involved in the regulation of cell proliferation. A. Fold change scatterplots. Cross species comparison of the fold changes of the genes in the pathway "Development, SSTR2 in regulation of cell proliferation" from GeneGo (see also Supplemental Table 3). The data were analyzed as described in Fig. 3A. **B.** Expression signal profile plots. Expression pattern of 3 genes. Mouse: blue; Rat: red; MO: Morula; ICM: Inner cell mass; BL: Blastocyst.

Suppl. Figure 5. Cross species analysis of the Wnt pathway. A. Fold change scatterplots. Cross species comparison of the fold changes of the genes in the pathway "Development, WNT signaling pathway. Part 2" from GeneGo (see also Supplemental Table 3). The data were analyzed as described in Fig. 3A. **B.** Expression signal profile plots. Expression pattern of 6 genes. Mouse: blue; Rat: red; MO: Morula; ICM: Inner cell mass; BL: Blastocyst.

Suppl. Figure 6. Cross species analysis of apoptotic processes. A. Fold change scatterplots. Cross species comparison of the fold changes of the genes in the pathway "Apoptosis and survival, NGF signaling pathway" from GeneGo (see also Supplemental Table 3). The data were analyzed as described in Fig. 3A. **B.** Expression signal profile plots. Expression pattern of 4 genes. Mouse: blue; Rat: red; MO: Morula; ICM: Inner cell mass; BL: Blastocyst.

Suppl. Figure 7. Cross species analysis of apoptotic processes. A. Fold change scatterplots. Cross species comparison of the fold changes of the genes in the pathway "Apoptosis and survival, Apoptotic Activin A signaling" from GeneGo (see also Supplemental Table 3). The data were analyzed as described in Fig. 3A. **B.** Expression signal profile plots. Expression pattern of 5 genes. Mouse: blue; Rat: red; MO: Morula; ICM: Inner cell mass; BL: Blastocyst.

Suppl. Figure 8. Cross species analysis of MAPK family. Fold change scatterplots. Scatterplots of the fold changes measured in the three comparisons for 12 members of the MAPK family in the mouse and in the rat. The complete list of all the genes analyzed as well as their fold changes are reported in the Supplemental Table 4.

Supplemental Tables

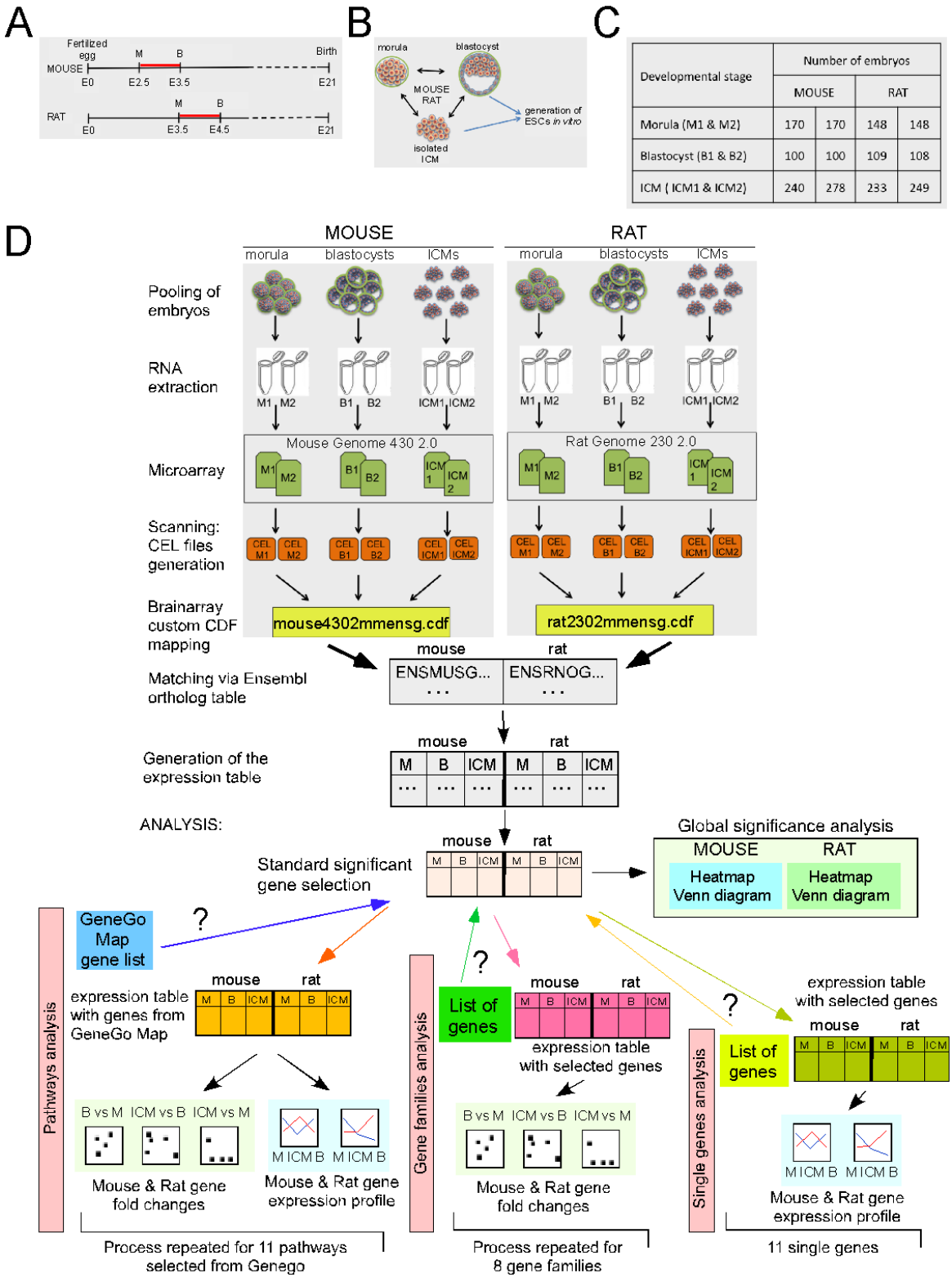
Suppl. Table 1. Mouse Venn diagram 1.5 fold change gene list. A. ICM versus Blastocyst. **B.** Blastocyst versus Morula. **C.** ICM versus Morula.

Suppl. Table 2. Rat Venn diagram 1.5 fold change gene list. A. ICM versus Blastocyst. **B.** Blastocyst versus Morula. **C.** ICM versus Morula.

Suppl. Table 3. Pathway analysis. List of genes and fold changes in the mouse and in the rat for the three comparisons (blastocyst versus morula, ICM versus blastocyst, ICM versus morula).

Suppl. Table 4. Gene family analysis. List of genes and fold changes in the mouse and in the rat for the three comparisons (blastocyst versus morula, ICM versus blastocyst, ICM versus morula).

Figure 1.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M
B
Birth
MOUSE
E0
E2.5
E3.5
E21
RAT
E0
E3.5
E4.5
E21
B
morula
blastocyst
MOUSE
RAT
generation of ESCs in vitro
isolated ICM
C
Developmental stage
Number of embryos
MOUSE
RAT
Morula (M1 & M2) 170 170 148 148
Blastocyst (B1 & B2) 100 100 109 108
ICM (ICM1 & ICM2) 240 278 233 249
D
MOUSE
RAT
morula
blastocysts
ICMs
Pooling of embryos
RNA extraction
M1 M2
B1 B2
ICM1 ICM2
Microarray
Mouse Genome 430 2.0
Rat Genome 230 2.0
Scanning: CEL files generation
Brainarray custom CDF mapping
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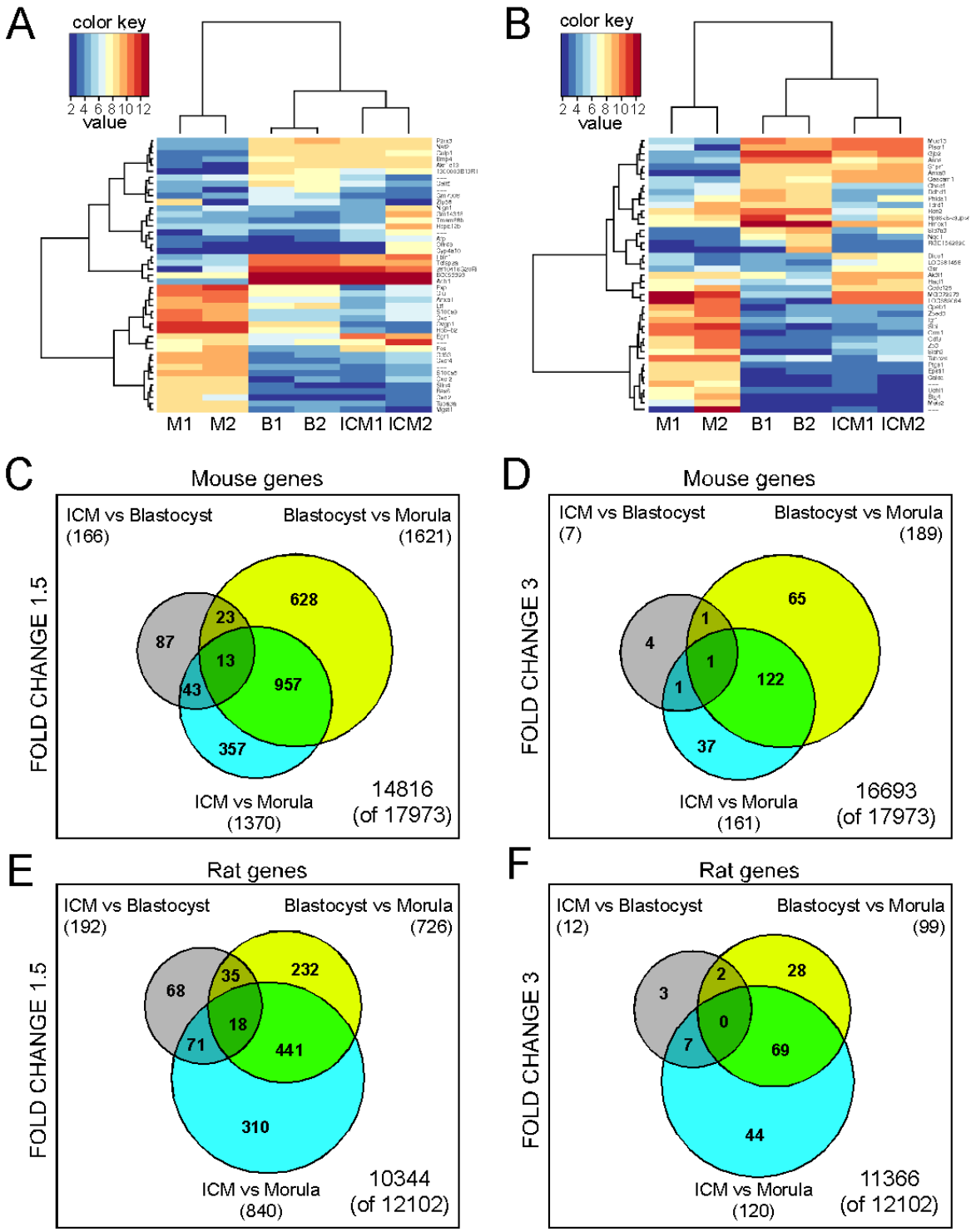
rat2302mmensg.cdf
Matching via Ensembl ortholog table
mouse
rat
ENSMUSG...
ENSRNOG...
Generation of the expression table
M B ICM M B ICM
ANALYSIS:
Standard significant gene selection
Global significance analysis
Heatmap Venn diagram
Pathways analysis
GeneGo Map gene list
expression table with genes from GeneGo Map
B vs M ICM vs B ICM vs M
M ICM B M ICM B
Mouse & Rat gene fold changes
Mouse & Rat gene expression profile
Process repeated for 11 pathways selected from Genego
Gene families analysis
List of genes
expression table with selected genes
Process repeated for 8 gene families
Single genes analysis
11 single genes

Figure 2.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color key
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value
M1 M2 B1 B2 ICM1 ICM2
B
color key
2 4 6 8 10 12
value
M1 M2 B1 B2 ICM1 ICM2
C
Mouse genes
ICM vs Blastocyst
(166)
Blastocyst vs Morula
(1621)
FOLD CHANGE 1.5
628
23
87
13
957
43
357
ICM vs Morula
(1370)
14816
(of 17973)
D
Mouse genes
ICM vs Blastocyst
(7)
Blastocyst vs Morula
(189)
FOLD CHANGE 3
65
1
4
1
122
1
37
ICM vs Morula
(161)
16693
(of 17973)
E
Rat genes
ICM vs Blastocyst
(192)
Blastocyst vs Morula
(726)
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35
68
18
441
71
310
ICM vs Morula
(840)
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Rat genes
ICM vs Blastocyst
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Blastocyst vs Morula
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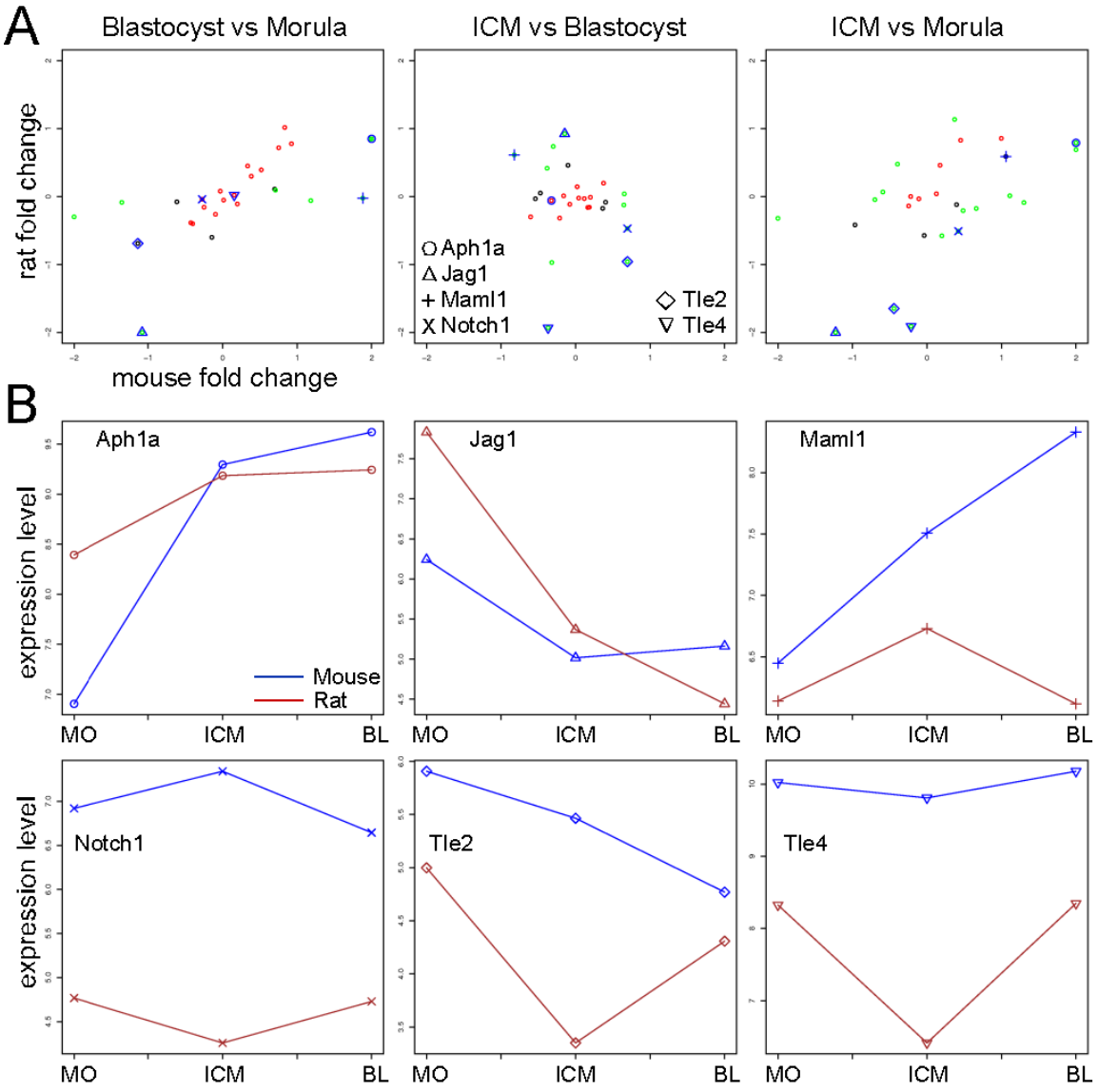
(of 12102)

Figure 3.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ICM vs Blastocyst
ICM vs Morula
rat fold change
mouse fold change
Aph1a
Jag1
Maml1
Notch1
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Tle4
B
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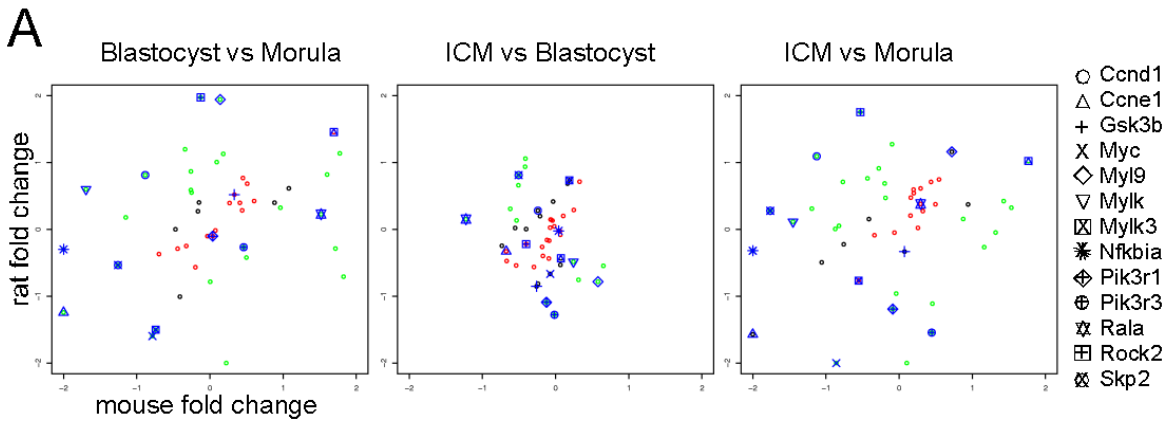
Maml1
Notch1
Tle2
Tle4
expression level
Mouse
Rat
MO
ICM
BL

Figure 4A.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Blastocyst vs Morula
ICM vs Blastocyst
ICM vs Morula
rat fold change
mouse fold change
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Ccne1
Gsk3b
Myc
Myl9
Mylk
Mylk3
Nfkbia
Pik3r1
Pik3r3
Rala
Rock2
Skp2

Figure 4B.

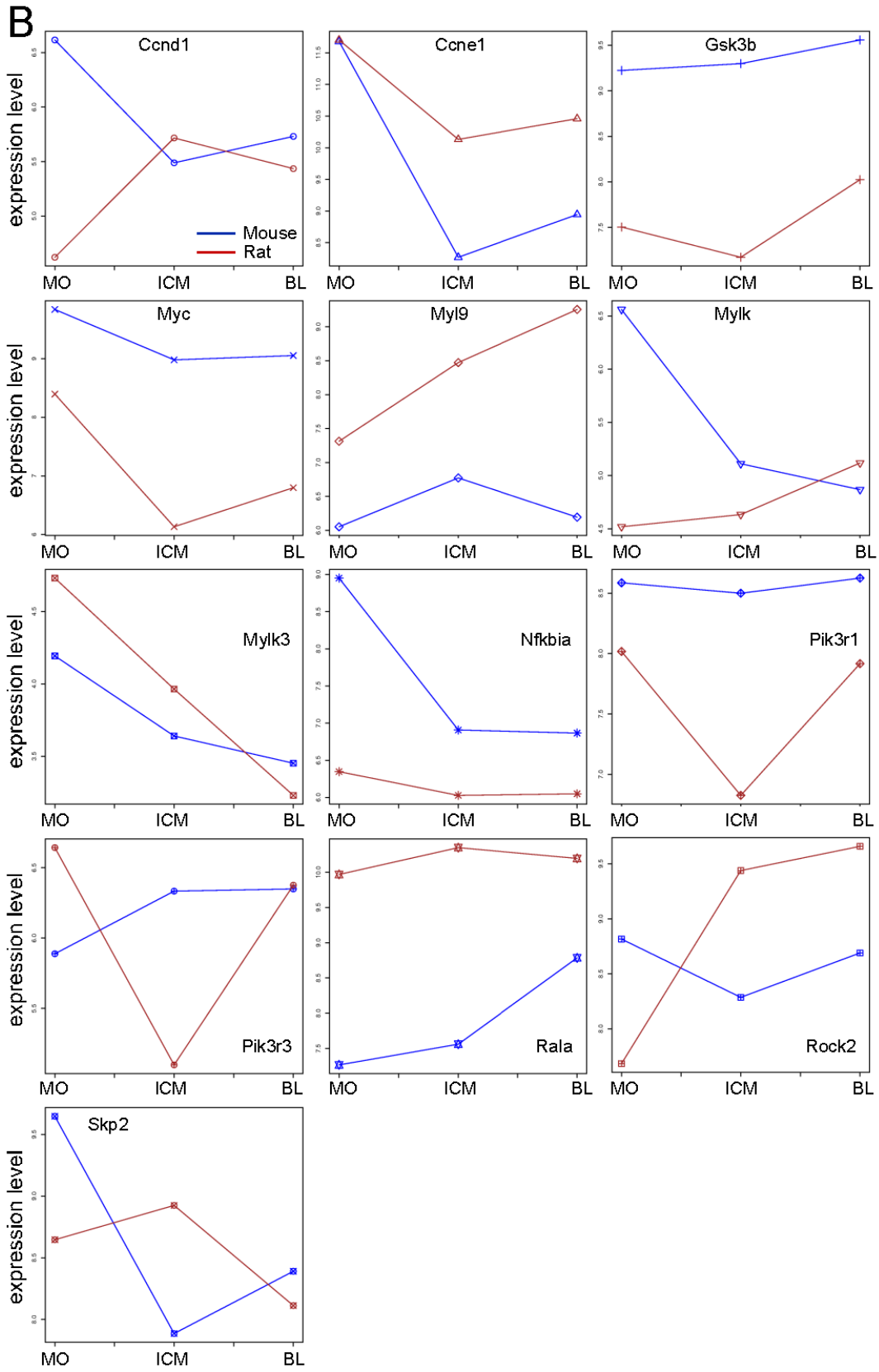

B
Ccnd1
Ccne1
Gsk3b
Myc
Myl9
Mylk
Mylk3
Nfkbia
Pik3r1
Pik3r3
Rala
Rock2
Skp2
Mouse
Rat
expression level
MO
ICM
BL

Figure 5.

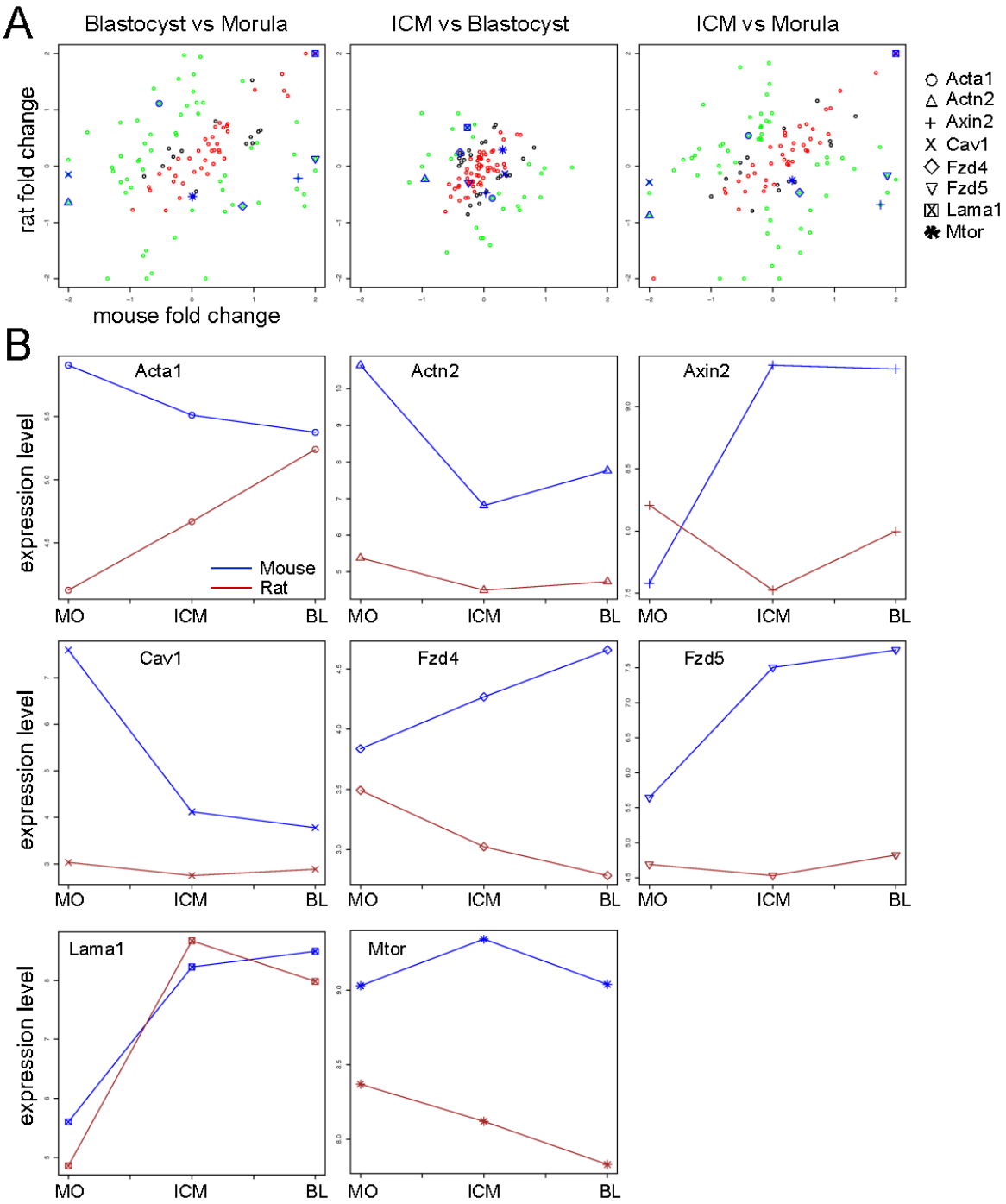
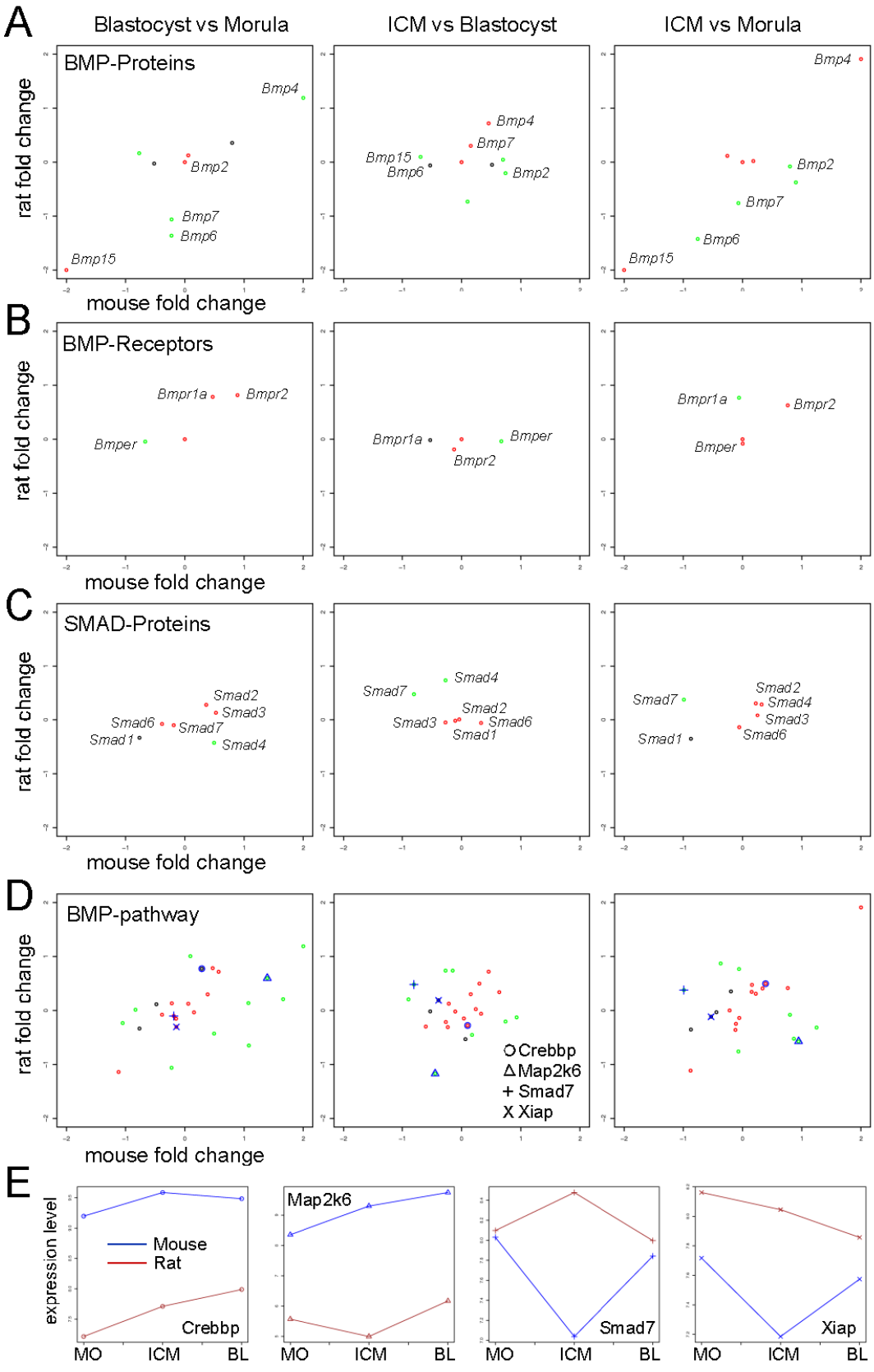

A
Blastocyst vs Morula
ICM vs Blastocyst
ICM vs Morula
rat fold change
mouse fold change
Acta1
Actn2
Axin2
Cav1
Fzd4
Fzd5
Lama1
Mtor
B
Acta1
Actn2
Axin2
Cav1
Fzd4
Fzd5
Lama1
Mtor
expression level
Mouse
Rat
MO
ICM
BL

Figure 6.


A
Blastocyst vs Morula
ICM vs Blastocyst
ICM vs Morula
BMP-Proteins
rat fold change
mouse fold change
Bmp4
Bmp2
Bmp7
Bmp6
Bmp15
B
BMP-Receptors
Bmpr1a
Bmpr2
Bmper
C
SMAD-Proteins
Smad2
Smad3
Smad6
Smad7
Smad1
Smad4
D
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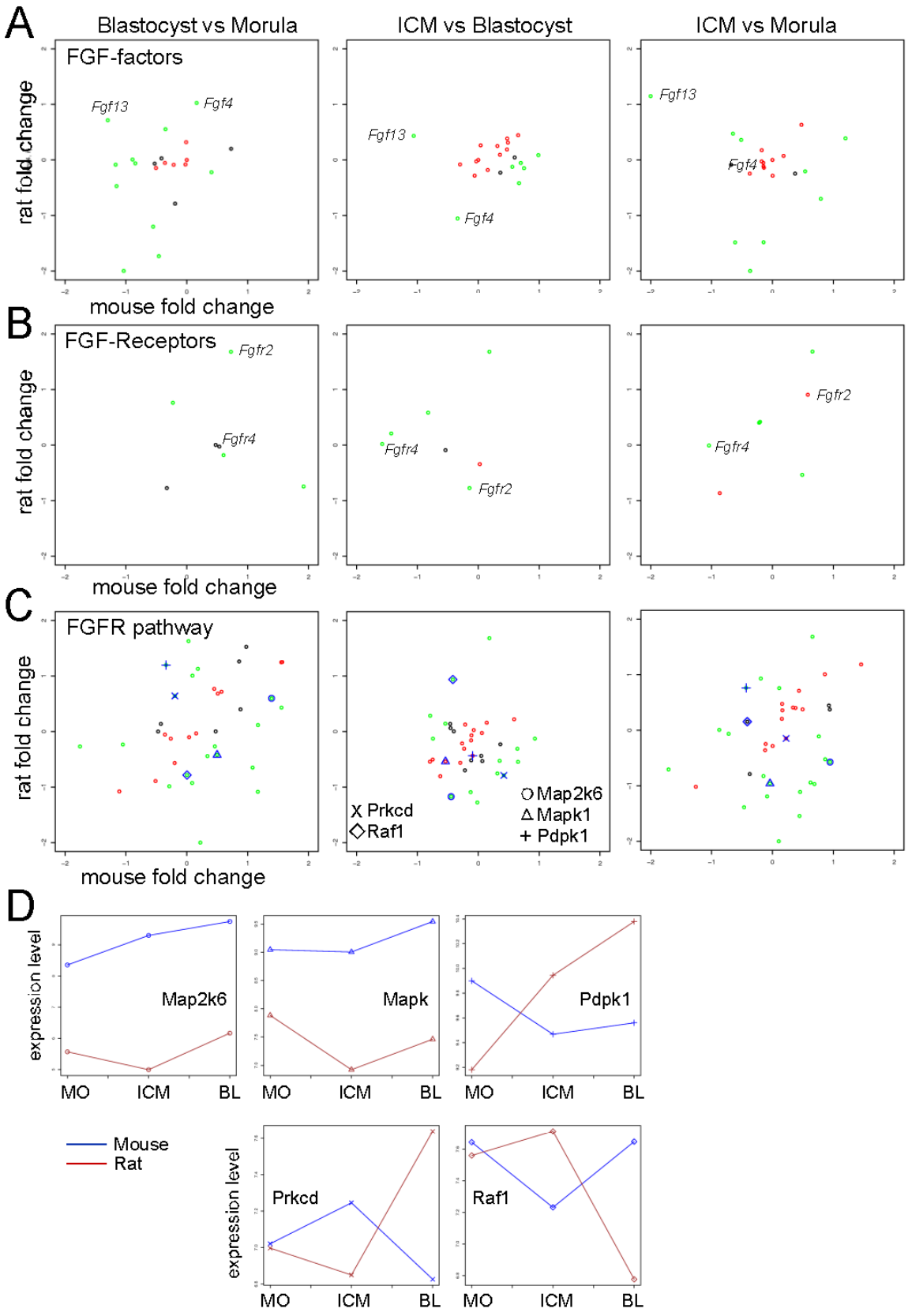
Map2k6
Smad7
Xiap
E
expression level
Mouse
Rat
MO
ICM
BL

Figure 7.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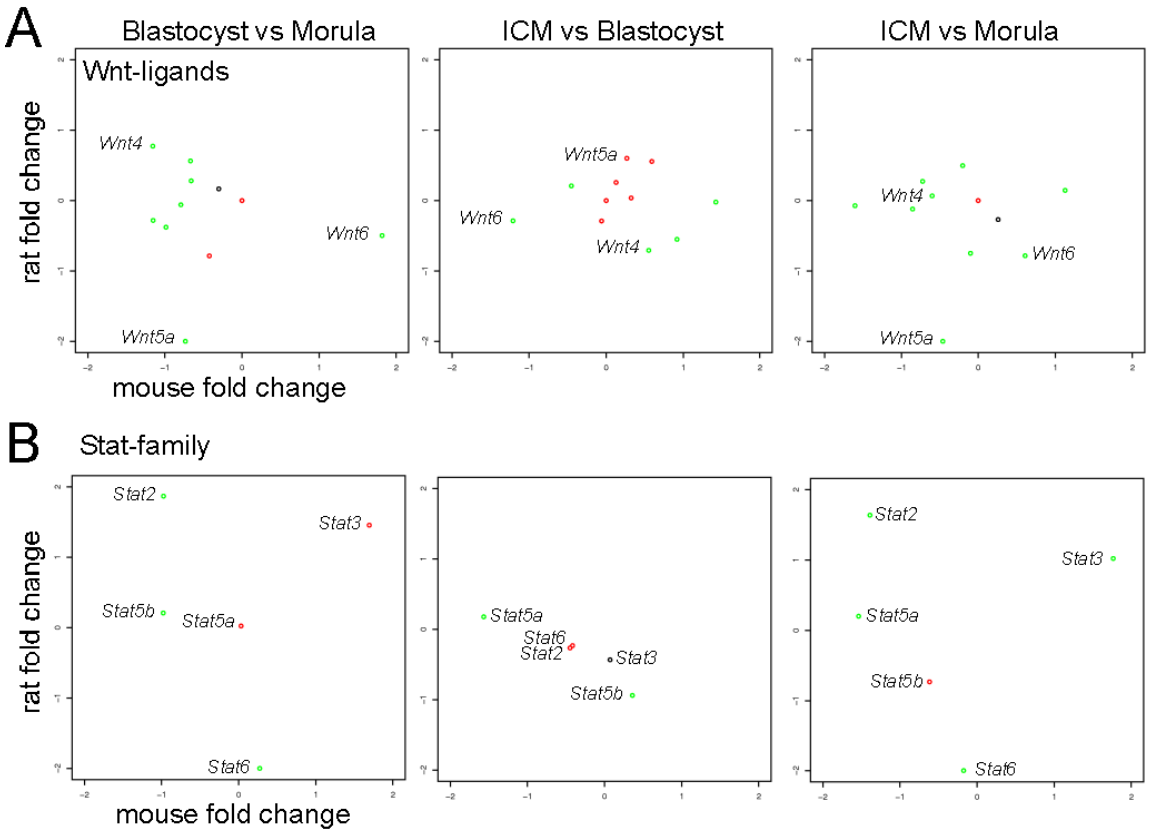
ICM vs Blastocyst
ICM vs Morula
FGF-factors
Fgf13
Fgf4
rat fold change
mouse fold change
B
FGF-Receptors
Fgfr2
Fgfr4
C
FGFR pathway
X Prkcd
◇Raf1
○Map2k6
△Mapk1
+ Pdpk1
D
expression level
Map2k6
Mapk
Pdpk1
MO
ICM
BL
Mouse
Rat
Prkcd
Raf1

Figure 8.


A
Blastocyst vs Morula
ICM vs Blastocyst
ICM vs Morula
Wnt-ligands
rat fold change
mouse fold change
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Wnt6
Wnt5a
B
Stat-family
Stat2
Stat3
Stat5b
Stat5a
Stat6

Figure 9.

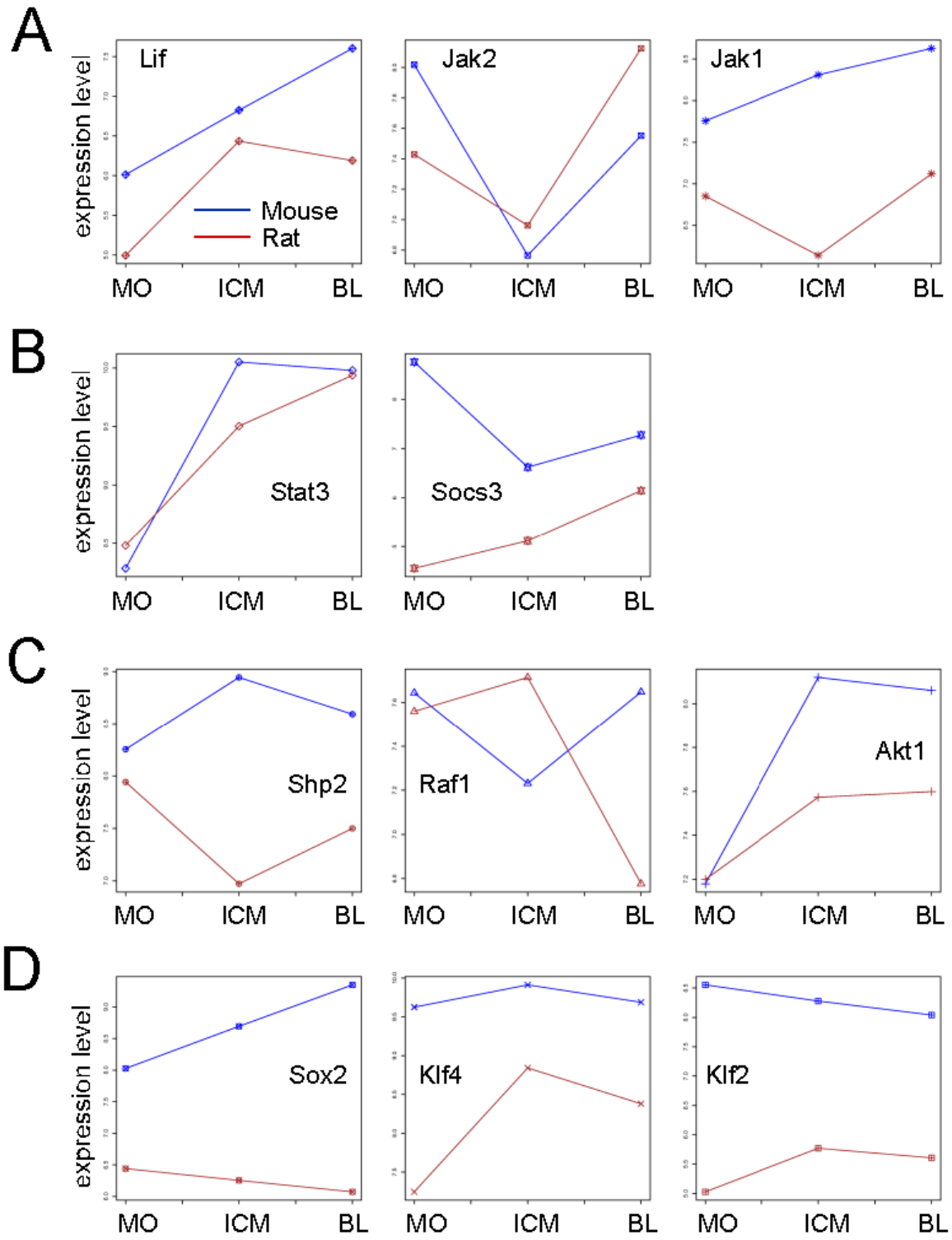
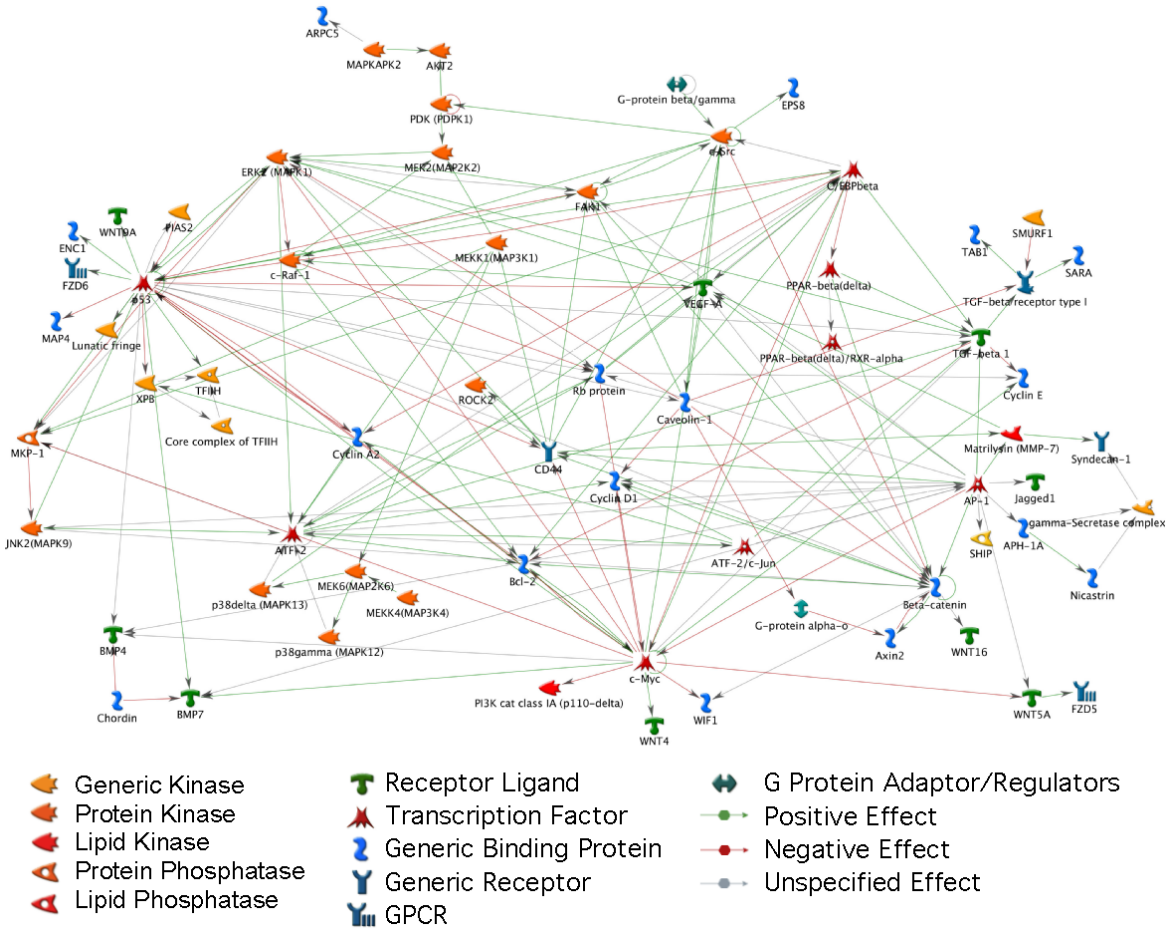
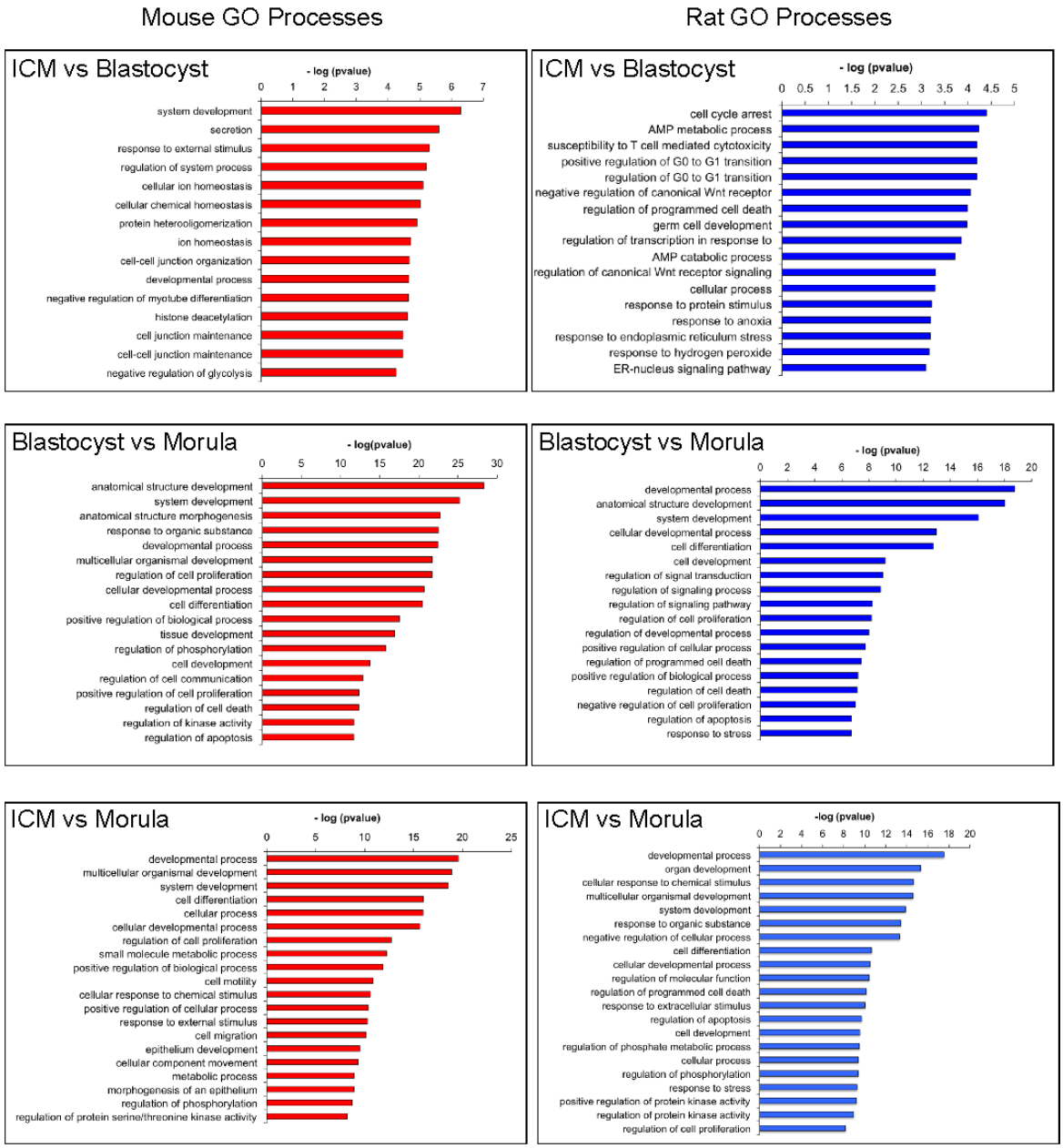

A
B
C
D
expression level
Lif
Jak2
Jak1
Mouse
Rat
Stat3
Socs3
Shp2
Raf1
Akt1
Sox2
Klf4
Klf2
MO
ICM
BL

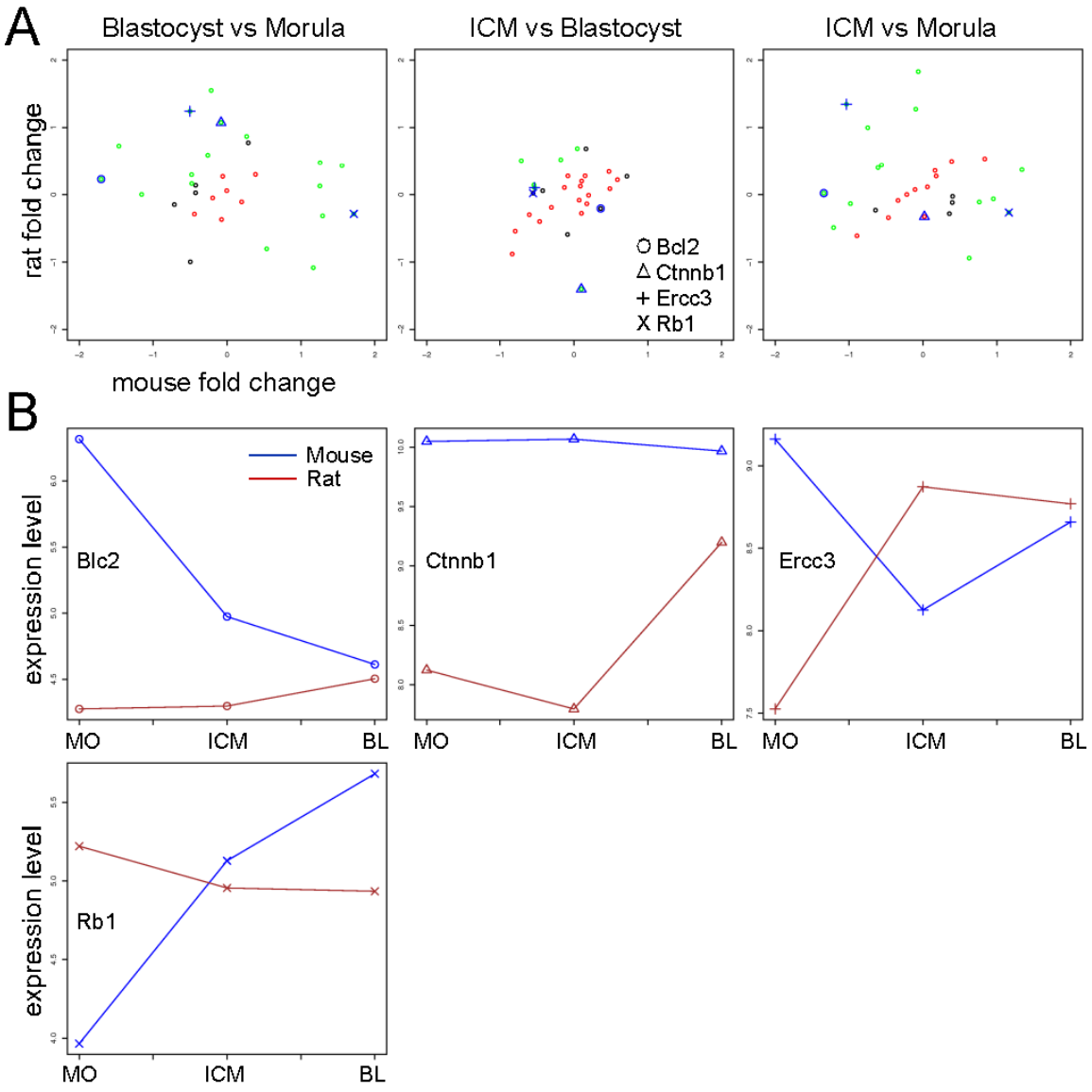
Figure 10.


ARPC5
MAPKAPK2
AKT2
PDK (PDPK1)
G-protein beta/gamma
EPS8
c-Src
ERK2 (MAPK1)
MEK2(MAP2K2)
FAK1
C/EBPbeta
WNT5A
PIAS2
ENC1
FZD6
p53
MAP4
Lunatic fringe
c-Raf-1
MEKK1(MAP3K1)
VEGF-A
PPAR-beta(delta)
PPAR-beta(delta)/RXR-alpha
TAB1
SMURF1
SARA
TGF-beta receptor type I
TGF-beta 1
Cyclin E
XPB
TFIIH
Core complex of TFIIH
ROCK2
Rb protein
Caveolin-1
MKP-1
Cyclin A2
CD44
Cyclin D1
Matrilysin (MMP-7)
Syndecan-1
Jagged1
AP-1
gamma-Secretase complex
APH-1A
SHIP
Nicastrin
JNK2(MAPK9)
ATF-2
MEK6(MAP2K6)
p38delta (MAPK13)
MEKK4(MAP3K4)
Bcl-2
ATF-2/c-Jun
Beta-catenin
G-protein alpha-o
Axin2
WNT16
BMP4
p38gamma (MAPK12)
c-Myc
PI3K cat class IA (p110-delta)
WIF1
WNT4
Chordin
BMP7
WNT5A
FZD5
Generic Kinase
Protein Kinase
Lipid Kinase
Protein Phosphatase
Lipid Phosphatase
Receptor Ligand
Transcription Factor
Generic Binding Protein
Generic Receptor
GPCR
G Protein Adaptor/Regulators
Positive Effect
Negative Effect
Unspecified Effect

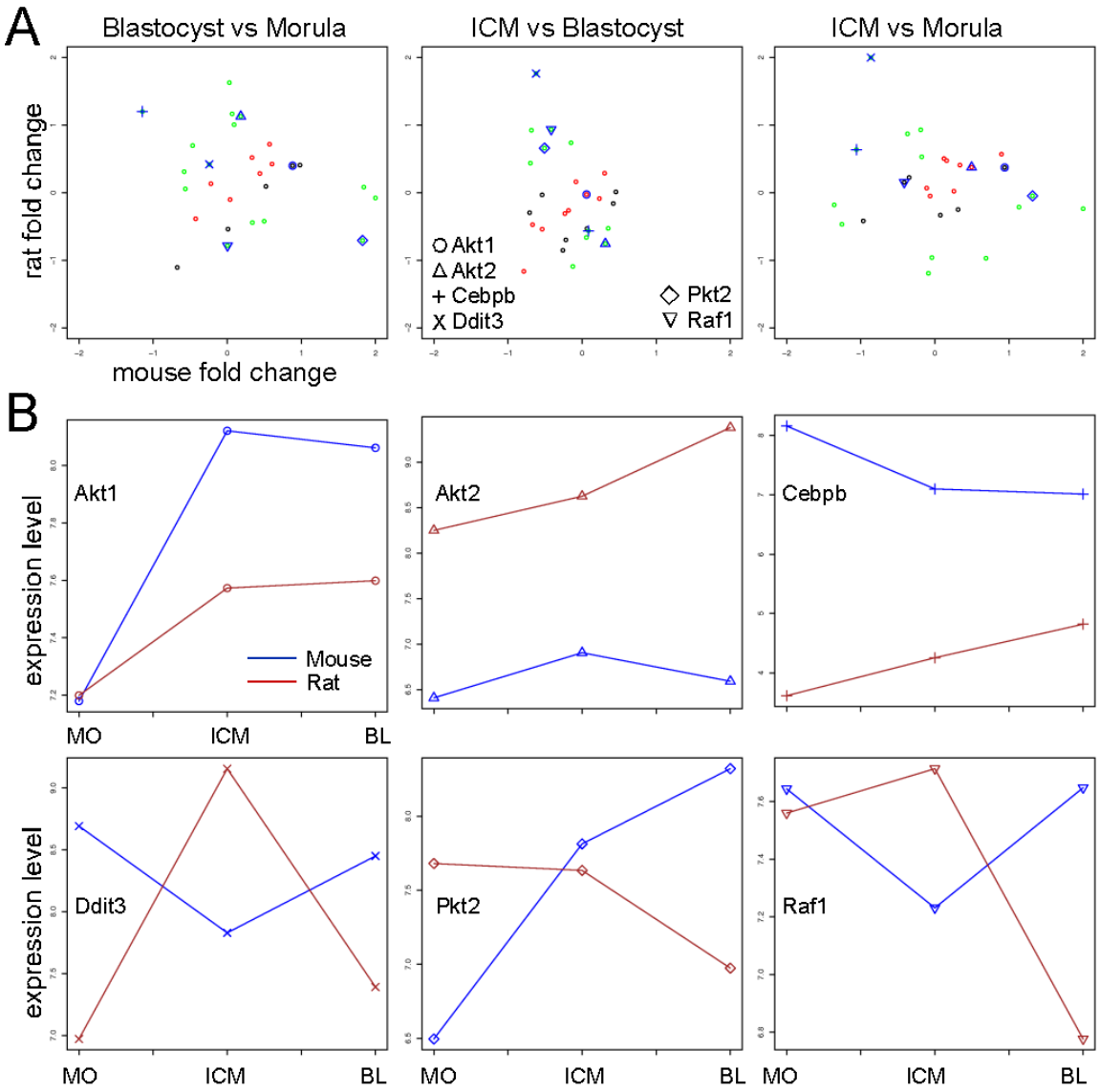
Supplemental Figure 1.



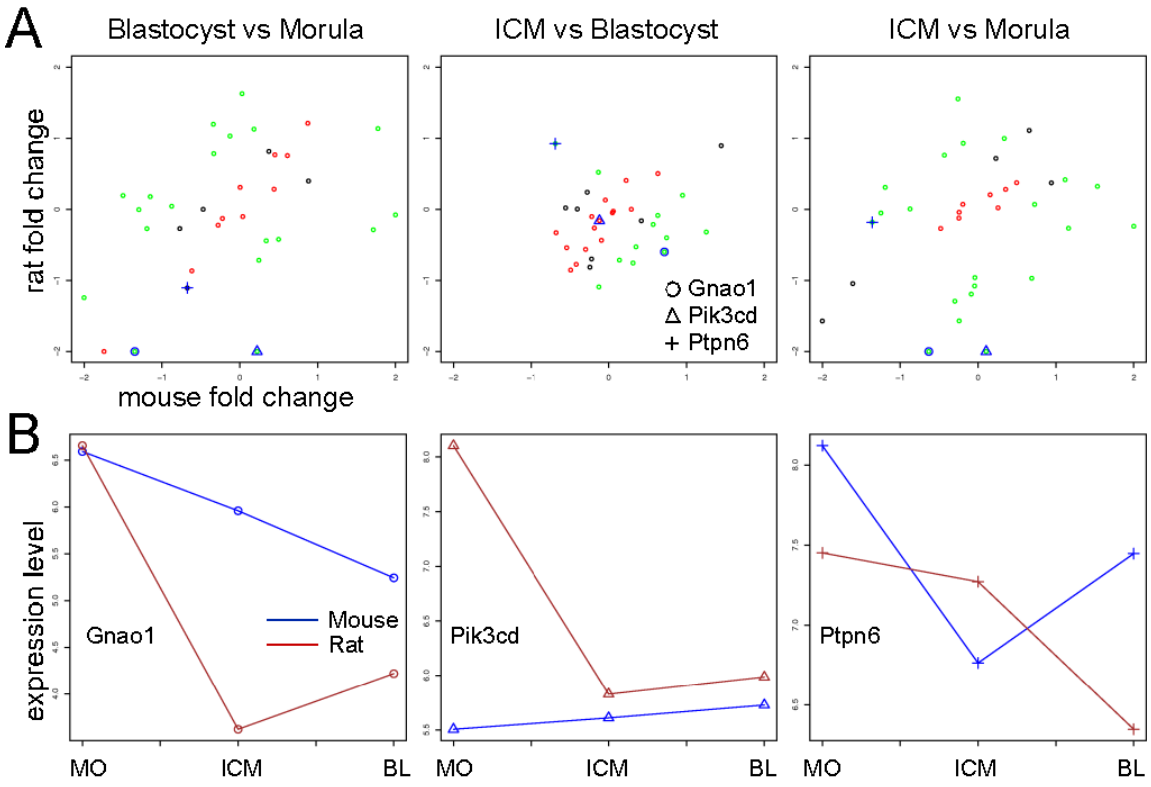
Supplemental Figure 2.



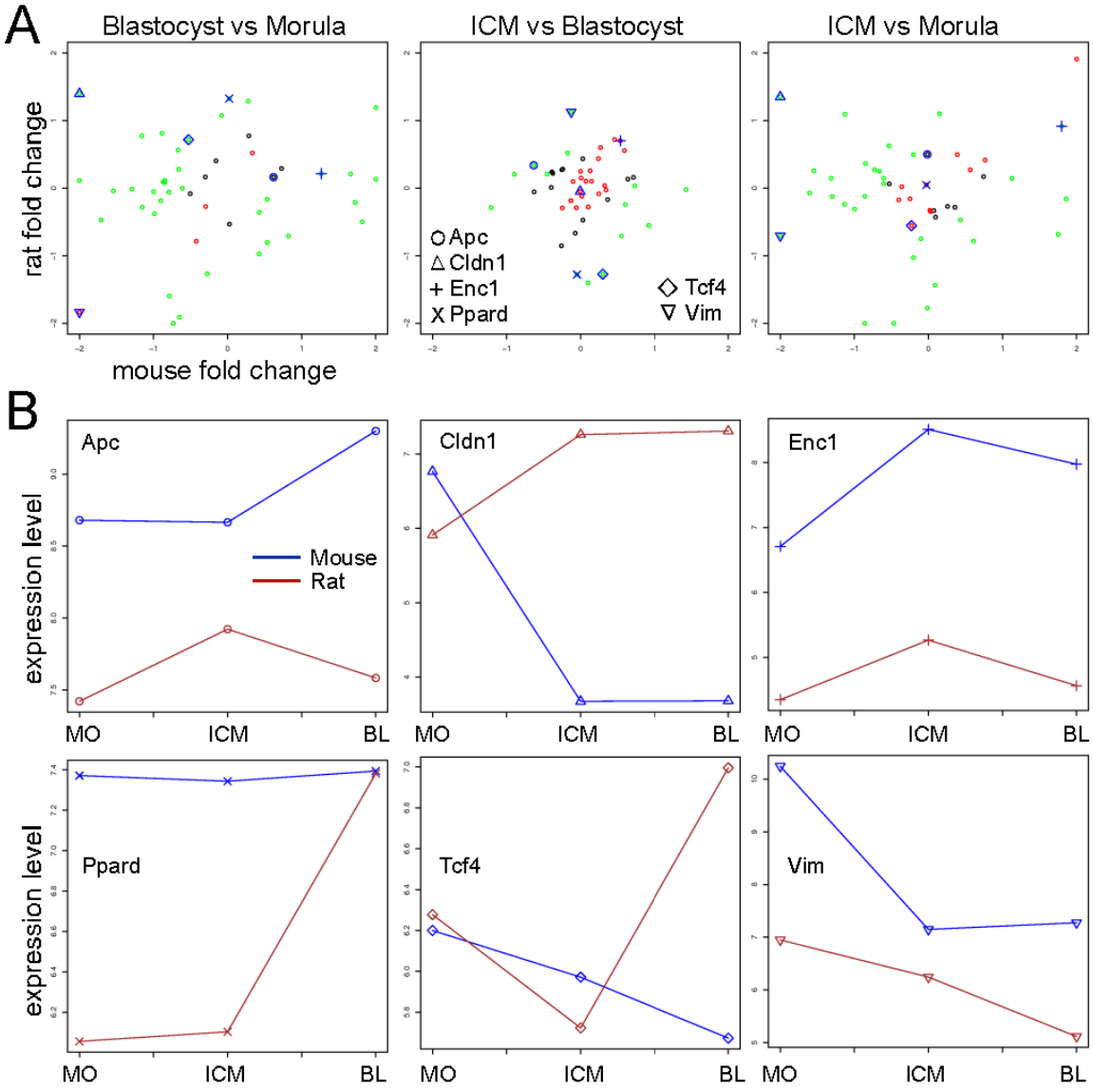
Supplemental Figure 3.



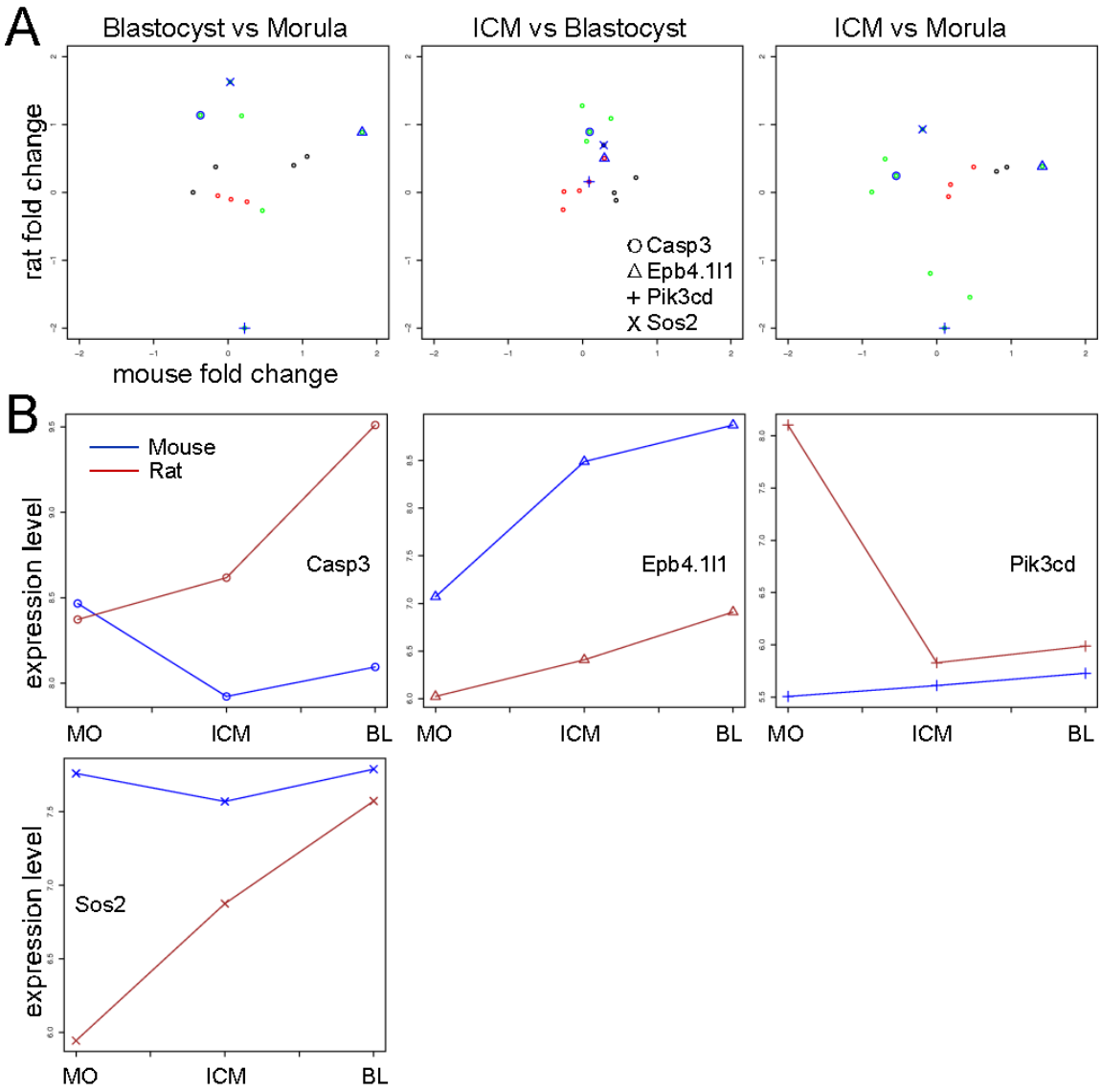
Supplemental Figure 4.



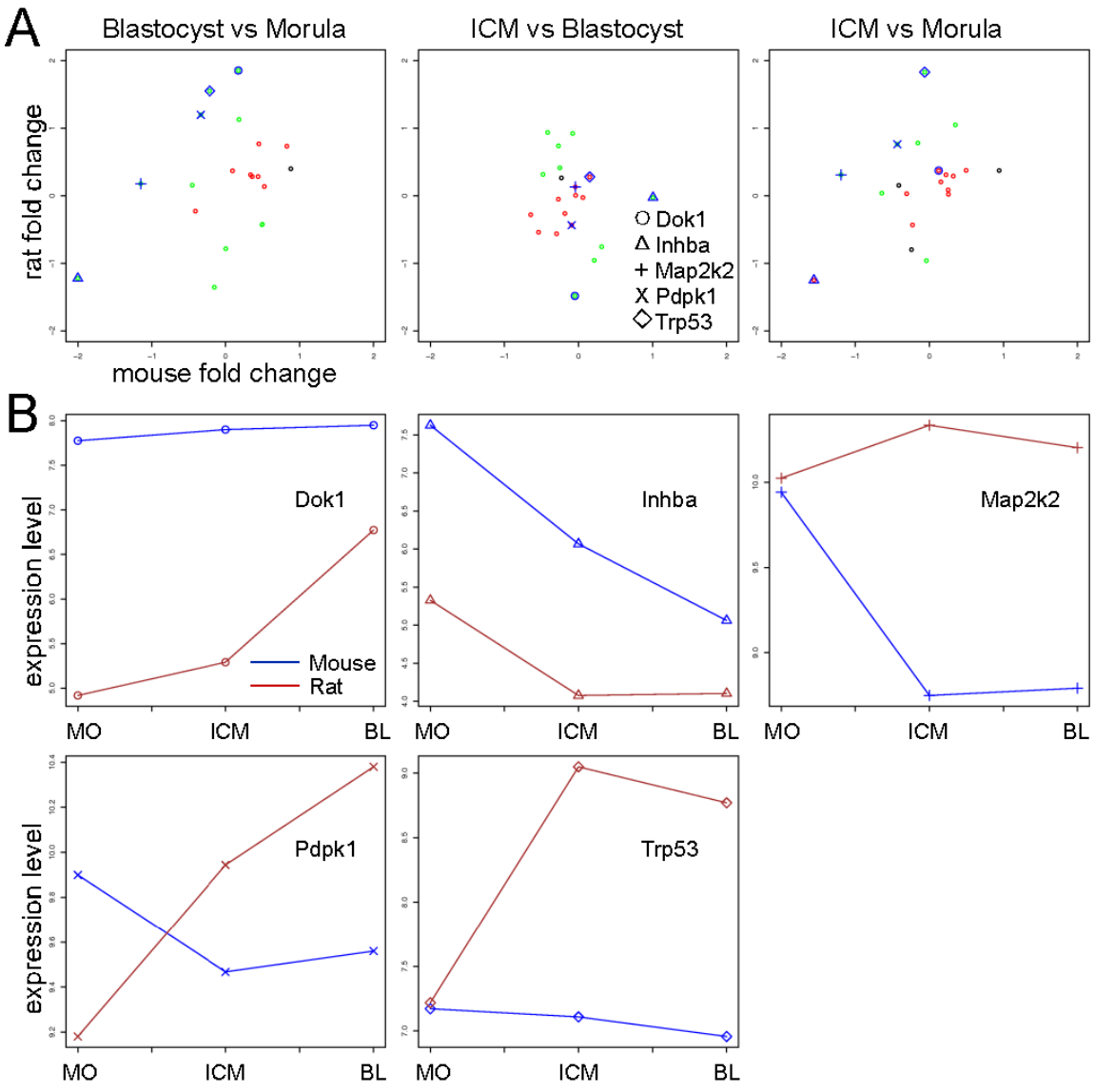
Supplemental Figure 5.



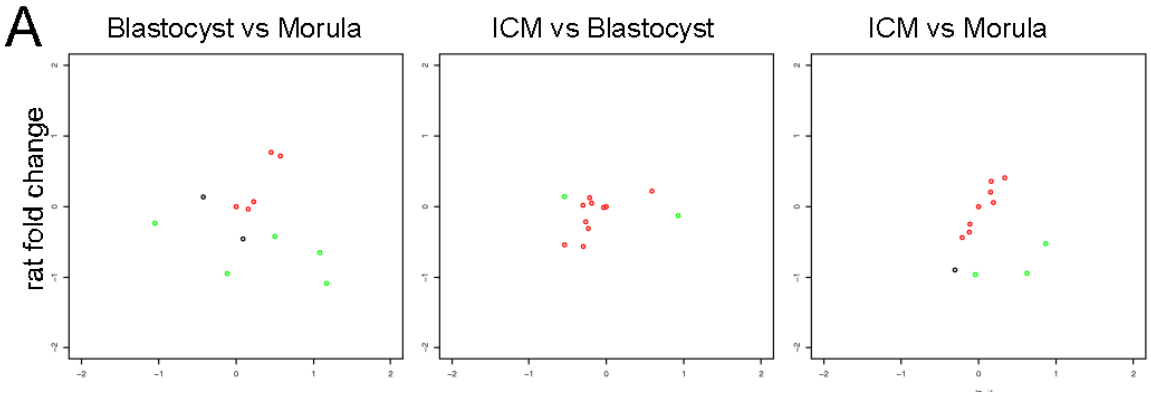
Supplemental Figure 6.



Supplemental Figure 7.



Supplemental Figure 8.



CURRICULUM VITAE

Elisa Casanova

Date of Birth : April 29th, 1983

Citizenship : Switzerland

Professional Experience

07/ 2007 – Today **PhD Student in Biology**
Institute for Laboratory Animal Science, University of Zurich
Supervisor: Dr. Paolo Cinelli

First Project: Comparison of pluripotency-related gene networks in mouse and rat
Second Project: Pramel7, a new determinant of pluripotency in embryonic stem cells

02/ 2006 – 04/ 2007 **Master Student in Biology**
Institute for Laboratory Animal Science, University of Zurich
Supervisor: Prof. K. Bürki and Dr. Paolo Cinelli

Research Theme: Functional characterization of newly identified genes related to pluripotency in embryonic stem cells

Education

07/ 2007 – Today	**PhD in Biology** University of Zurich, Switzerland
09/ 2005 – 04/ 2007	**Master of Science in Human Biology** University of Zurich, Switzerland
09/ 2002 – 06/ 2005	**Bachelor of Science in Biology** University of Zurich, Switzerland
09/ 1998 – 06/ 2002	**High school education** Liceo Federale Diocesano Pio XII, Lugano, Switzerland General Certificate of Secondary Education: focus on languages (German, English and French)

Languages

Italian:	Mother tongue
English:	Excellent
German:	Excellent
French:	Excellent

Publications

Cinelli P, Casanova EA, Uhlig S, Lochmatter P, Matsuda T, Yokota T, Rülicke T, Ledermann B, Bürki K. (2008) Expression profiling in transgenic FVB/N embryonic stem cells overexpressing STAT3. BMC Dev Biol, 8, 57.

Casanova EA, Shakhova O, Patel S, Asner I, Pelczar P, Weber FA, Graf U, Sommer L, Bürki K, Cinelli P. (2011) Pramel7 mediates LIF/STAT3 dependent self-renewal in embryonic stem cells *Stem Cells* 29(3):474-85.

Casanova EA, Bürki K, Cinelli P. (2011) Molecular mechanisms of pluripotency in "Embryonic Stem Cells: The Hormonal Regulation of Pluripotency and Embryogenesis" ISBN 978-953-307-196-1. INTECH, *in press*.

Graf U, Casanova EA, Cinelli P. (2011) The role of the LIF-pathway in derivation and maintenance of murine pluripotent stem cells. Genes 2, 280-297.

Casanova EA, Okoniewski MJ, Cinelli P. (2011) Cross-species genome wide expression analysis of late mouse and rat preimplantation embryos. *Submitted*

Posters

Elisa A. Casanova, Olga Shakhova, Lukas Sommer, Kurt Bürki and Paolo Cinelli (2011) Pramel7 mediates LIF/STAT3 dependent self-renewal in embryonic stem cells. EMBO Conference: Advances in Stem Cell Research, Institute Pasteur, Paris, France.

Akshay Ahuja, **Elisa Casanova**, Daniel Zingg, Hitoshi Takizawa, Markus Manz, Lukas Sommer, Paolo Cinelli and Massimo Lopes (2011) DNA damage response (DDR) in stem cells: at the crossroad of ageing, cancer therapy and regenerative medicine. Brupbacher Symposium: Cancer Genome and DNA Repair, Zurich, Switzerland.

Elisa A. Casanova, Olga Shakhova, Lukas Sommer, Kurt Bürki and Paolo Cinelli (2011) Pramel7 mediates LIF/STAT3 dependent self-renewal in embryonic stem cells. 7th annual Swiss Stem Cell Network Meeting, EPFL Lausanne, Switzerland

Elisa A. Casanova, Olga Shakhova, Lukas Sommer, Kurt Bürki and Paolo Cinelli (2011) Pramel7 mediates LIF/STAT3 dependent self-renewal in embryonic stem cells. USGEB Meeting, Zürich, Switzerland: P-33 (selected for oral presentation)

Elisa A. Casanova, Olga Shakhova, Lukas Sommer, Kurt Bürki and Paolo Cinelli (2010) Pramel7: A New Determinant of Pluripotency in Embryonic Stem Cells. 6th annual Swiss Stem Cell Network Meeting, University of Basel, Switzerland.

Elisa A. Casanova, Olga Shakhova, Lukas Sommer, Kurt Bürki and Paolo Cinelli (2009) Pramel7: A New Determinant of Pluripotency in Embryonic Stem Cells.
Gordon Research Conference on Stem Cells & Cancer, Les Diablerets, Switzerland.

Paolo Cinelli, Syndi Uhlig, Priska Lochmatter, **Elisa Casanova**, Kurt Bürki (2007) Expression profiling in transgenic FVB/N embryonic stem cells overexpressing STAT3. Swiss Stem Cell Network 4th Annual Meeting, Zürich, Switzerland: 7.

Paolo Cinelli, Syndi Uhlig, Priska Lochmatter, **Elisa Casanova**, Kurt Bürki (2007) Expression profiling in transgenic FVB/N embryonic stem cells overexpressing STAT3. EMBL Conference on Functional Genomics with Embryonic Stem Cells, EMBL Heidelberg, Germany: 46.

Talks at congresses

Elisa Casanova (2011) "Pramel7 mediates LIF/STAT3 dependent self-renewal in embryonic stem cells", USGEB Meeting, Zurich, Switzerland.